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Therapeutic targeting of cathepsin C: from pathophysiology to treatment

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Therapeutic Targeting of Cathepsin C: *from pathophysiology to treatment*

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Abstract

Cathepsin C (CatC) is a highly conserved tetrameric lysosomal cysteine dipeptidyl aminopeptidase. The best characterized physiological function of CatC is the activation of pro-inflammatory granule-associated serine proteases. These proteases are synthesized as inactive zymogens containing an N-terminal pro-dipeptide, which maintains the zymogen in its inactive conformation and prevents premature activation, which is potentially toxic to the cell. The activation of serine protease zymogens occurs through cleavage of the N-terminal dipeptide by CatC during cell maturation in the bone marrow. *In vivo* data suggest that pharmacological inhibition of pro-inflammatory serine proteases would suppress or attenuate deleterious effects of inflammatory/auto-immune disorders mediated by these proteases. The pathological deficiency in CatC is associated with Papillon-Lefèvre syndrome. The patients however do not present marked immunodeficiency despite the absence of active serine proteases in immune defense cells. Hence, the transitory pharmacological blockade of CatC activity in the precursor cells of the bone marrow may represent an attractive therapeutic strategy to regulate activity of serine proteases in inflammatory and immunologic conditions. A variety of CatC inhibitors have been developed both by pharmaceutical companies and academic investigators, some of which are currently being employed and evaluated in preclinical/clinical trials.

Key words: cathepsin C, serine proteases, Papillon-Lefèvre syndrome, inflammatory/autoimmune diseases, therapeutic inhibitors, pharmacological targeting

Abbreviations: AAT, α1-antitrypsin; AATD, α1-antitrypsin deficiency; ANCA, anti-neutrophil cytoplasmic autoantibody; AAV, ANCA-associated vasculitis; Cat, cathepsin; COPD, chronic obstructive pulmonary disease, GPA, granulomatosis with polyangiitis; HMS, Haim-Munk syndrome; IL, interleukin; MMP, matrix metalloprotease; MPO, myeloperoxidase; NCGN, necrotizing crescengic glomerulonephritis; MPA, microscopic polyangiitis, NE, neutrophil elastase; NETs, neutrophil extracellular traps; NSP, neutrophil serine protease; PLS, Papillon-Lefèvre syndrome; PPK, palmo-

plantar keratoderma; PR3, proteinase 3, mPR3, membrane-bound PR3; MIP1α, macrophage inhibitory protein-1 alpha; ROS, reactive oxygen species.

1. INTRODUCTION

Complex type serine proteases of coagulation and clotting system and the simple digestive proteases of the pancreas are synthesized and either constitutively secreted or stored as inactive precursors (zymogens) in cellular granules. In response to specific stimuli, these zymogens are locally and transiently converted to their active forms by strictly regulated limited proteolysis. By contrast, immune defense cells including neutrophils, mast cells, lymphocytes and macrophages, express a unique subset of eleven single domain serine proteases whose zymogens are already constitutively converted to their active form by cathepsin C (CatC) during biosynthesis, sorting and storage in cytoplasmic granules.

CatC, also known as dipeptidyl peptidase 1 (DPP1, EC 3.4.14.1), is a ubiquitously expressed lysosomal cysteine exopeptidase belonging to the papain family of cysteine peptidases (Turk, et al., 2001). It was discovered by Gutman and Fruton (Gutmann & Fruton, 1948). CatC cleaves two residues from the N-termini of proteins and peptides and is considered to be a major intracellular processing enzyme. CatC has an essential role in the activation of various granule serine proteases from neutrophils (elastase (NE), cathepsin G (CatG), proteinase 3 (PR3) and neutrophil serine protease 4 (NSP4)), from mast cells (chymase and tryptase), from cytotoxic T-lymphocytes and natural killer cells (granzymes A and B). Once activated these pro-inflammatory proteases can degrade various extracellular matrix compounds, leading to tissue damage and triggering chronic inflammation. The main physicochemical characteristics of CatC are listed in **Table 1**.

Based on preclinical/clinical data illustrating the pathological functions of granule-associated serine proteases, it appears that CatC represents an attractive therapeutic target for chronic inflammatory and auto-immune diseases (Korkmaz, Horwitz, Jenne, & Gauthier, 2010; Korkmaz, Lesner, et al., 2013). The finding that CatC knock-out mice are resistant to the detrimental actions of granule-associated serine proteases support the therapeutic strategy of pharmacological CatC inhibition (**Table 2**). Individuals with loss-of-function mutations of the CatC gene suffer from pre-pubertal aggressive periodontitis, Papillon-Lefèvre syndrome (PLS) (Toomes, et al., 1999) or Haim-Monk syndrome (Haim & Munk, 1965). These patients however do not exhibit marked

immunodeficiency despite the almost total absence of proteolytically active granule-associated serine proteases in immune defense cells (Pham, Ivanovich, Raptis, Zehnbauer, & Ley, 2004).

In this review, we first provide an overview of CatC, its functional biochemical properties and the consequences of its deficiency, e.g. in PLS. We then focus on the pathophysiology of serine proteases activated by CatC. Finally, we describe various CatC inhibitors, which are currently being evaluated in preclinical and clinical trials. This review was initiated following the first International Symposium *ISyCatC* on therapeutic targeting of CatC (Tours/France, April, 2017), which has brought together, for the first time, worldwide specialists from academic labs and industry working in the field.

2. STRUCTURE, ACTIVITY AND CELLULAR ROADMAP OF CATC

2.1 Cysteine cathepsins

2.1.1 Proteolytic enzymes: a brief opening

The preservation of the homeostatic regulation of organisms largely relies upon controlled biosynthesis, maturation, modulation of biological functions, and terminal proteolytic recycling of proteins. Proteases (also called peptidases or proteinases) are encoded by less than 2% of the human genome and form the largest family of human enzymes (Puente, Sanchez, Overall, & Lopez-Otin, 2003). There are almost 600 members, well ahead of the kinases (more than 450 members). Conversely, the human genome also encodes more than 200 endogenous protease inhibitors (Rawlings, Barrett, & Finn, 2016). Ultimately, 5-10% of proteases may be considered as valid therapeutic targets (Drag & Salvesen, 2010). Proteases are hydrolases the same as esterases and phosphatases. They may be divided into endopeptidases, which cleave irreversibly internal peptide (amide) bonds, and exopeptidases (including both aminopeptidases and carboxypeptidases). However, there are also distinct classes of proteases, each with its distinct mechanism of catalysis: metalloproteases, cysteine, serine, threonine, and aspartic proteases. Recently, a sixth class of proteases, so called glutamic (i.e. acidic like aspartic) proteases was identified, but members of this

class have not been found in mammals so far (Lopez-Otin & Bond, 2008; Rawlings, et al., 2016). The cysteine, serine, and threonine proteases all use an amino acid residue as a nucleophile (Cys, Ser, or Thr) located in their active site to attack the amide bond of the substrate. The other classes of proteases use a coordinated, activated water molecule as a nucleophile (Turk, 2006). Proteases are further grouped in families, based on similarities in their amino acid sequences. Families that are believed to be homologous because of similarities in tertiary structures are also grouped together to form clans (Rawlings & Barrett, 1993). The activities of proteases may be controlled at multiple molecular and cellular levels. This includes gene transcription, post-translational modifications (e.g. glycosylation and phosphorylation, metal/ion binding, disulfide - or persulfide - bridging), maturation of their zymogens (inactive proforms) by autocatalytic and heterocatalytic mechanisms, substrate-driven allosteric activation, oxidation, segregation within subcellular compartments, physicochemical environment (pH, charged heteropolysaccharides), ectodomain shedding (release of extracellular domains of integral membrane proteins) and, obviously, regulation of their proteolytic activity by protein and peptide inhibitors. It is noteworthy that, besides the “true” proteases that cleave alpha-peptide bonds, some proteolytic enzymes act as isopeptidases by cleaving isopeptide bonds (e.g. SENPS for "SENtrin-specific Proteases" that are deSUMOylating enzymes, or DUBs that are deubiquitylating enzymes) (Drag & Salvesen, 2008; Harrigan, Jacq, Martin, & Jackson, 2018).

2.1.2 Lysosomal cysteine cathepsins: some selected general features

The “catheptic activity” that is derived from the Greek word "kathépsein" (meaning: to digest or to boil down) was first described in the acidic fluid secreted by the stomach glands during the 1920s. Unfortunately, the inclusive meaning of the word "cathepsin" has caused confusion for many years. Several cathepsins, for example CatA and CatG (serine proteases) or CatD and CatE (aspartic proteases), are digestive enzymes but are unrelated to the cysteine cathepsins, the largest family that refer to family C1 (clan CA). There are eleven members in humans [i.e. cathepsins B, H, L, S, C, K, O, F, V, X (Z) and W (a.k.a. lymphopain)] and all cysteine cathepsins except cathepsin V have orthologs in mice (Turk, et al., 2012). They are closely related to papain, an extensively studied plant enzyme and the archetypal structural model. Cathepsins have relatively broad, overlapping

specificities, supporting the contention that they may partly exhibit functional redundancy (Nagler & Menard, 2003). They have also long been regarded as ubiquitous household enzymes, primarily involved in the recycling and degradation of proteins in lysosomes (Lecaille, Kaleta, & Bromme, 2002). Nevertheless, this opinion has changed extensively with the demonstration of their distinctive involvement in various specific biological processes and their identification as specific biomarkers of diseases (Fonovic & Turk, 2014a, 2014b). Indeed cysteine cathepsins contribute to highly specialized processes such as histone proteolysis during stem cell differentiation, maturation of neuropeptides and thyroid hormones, bone resorption, matrix remodeling, pulmonary homeostasis, hair cycle control and antigen presentation (Chapman, Riese, & Shi, 1997; Honey & Rudensky, 2003; Hook, et al., 2008; Kramer, Turk, & Turk, 2017; Lalmanach, Diot, Godat, Lecaille, & Herve-Grepinet, 2006; Taggart, et al., 2017; Vasiljeva, et al., 2007). Cathepsins are also involved in an extensive range of disorders (e.g. atherosclerosis, cardiomyopathy, adiposity, osteoporosis, rheumatoid arthritis, metastasis, fibrosis, emphysema, asthma or neuropathic pain) (Kramer, et al., 2017; Lafarge, Naour, Clement, & Guerre-Millo, 2010; Lalmanach, Saidi, Marchand-Adam, Lecaille, & Kasabova, 2015; Mohamed & Sloane, 2006; Olson & Joyce, 2015; Reiser, Adair, & Reinheckel, 2010; Vasiljeva & Turk, 2008). Therefore, even though their exact pathophysiological role is still often misunderstood, these observations make some of them, including CatC, attractive targets for new anti-protease drugs (Caughey, 2016; Drag & Salvesen, 2010; Korkmaz, Lesner, et al., 2013; Kramer, et al., 2017; Lecaille, Bromme, & Lalmanach, 2008; Turk, 2006; Wilkinson, Williams, Scott, & Burden, 2015). Recently developed chemical and biological tools as well innovative analytical methods will help to clarify the molecular mechanisms as well dysfunctions that occur during such pathophysiological events (Deu, Verdoes, & Bogyo, 2012; Hughes, Burden, Gilmore, & Scott, 2016; Sanman & Bogyo, 2014; Serim, Haedke, & Verhelst, 2012).

Sequences: Mammalian papain-like proteases are relatively small enzymes with a molecular mass in the range of 20-35 kDa, with the notable exception of CatC (molecular mass of the mature tetrameric protease is around 200 kDa) (Lecaille, et al., 2002; Turk, et al., 2012). Cysteine cathepsins have in common a signal peptide, a propeptide, and a catalytic domain with the latter representing the mature proteolytically active form (Cygler & Mort, 1997; Turk, Turk, & Turk, 2000). Signal peptides

are on average between 10 and 20 amino acids in length. Human propeptides are of variable length between 36 amino acids in CatX and 251 amino acids in CatF, while the catalytic domains are between 220 and 260 amino acids in length (Lecaille, et al., 2002; Nagler & Menard, 1998). The catalytic active site, which has some similarities to the active site present in serine proteases (Ser, His, Asp), is highly conserved and formed by three residues: Cys25, His159, and Asn175 (papain numbering) (Turk, Turk, & Turk, 2012). In contrast to some related parasite enzymes mammalian, cysteine cathepsins do not encompass a C-terminal extension of unknown function (Lalmanach, et al., 2002; Sajid & McKerrow, 2002). A unique feature of CatB is the insertion of an "occluding loop" between the conserved Pro-Tyr motif and Cys128 (CatB numbering) (Illy, et al., 1997). The loop is characterized by two adjacent histidine residues (His110, His111) that are responsible for the dipeptidyl carboxypeptidase activity of CatB (Mort & Buttle, 1997). Protein sequence alignments of catalytic domains reveal a clustering (based on the general degree of sequence identity and similarity among the proteases) into three major subfamilies: CatB-like, CatL-like, and CatF-like. Moreover, the affiliation to the three subfamilies is also revealed in a conserved motif within the proregions that have a significant lower degree of sequence similarity than the catalytic domains. The ERF/WNIN motif is characteristic for the CatL-like subfamily, but it is absent in CatB-like proteases and modified into an ERFNAQ/A motif in the CatF-like subfamily (P. J. Berti & Storer, 1995; Karrer, Peiffer, & DiTomas, 1993; Wex, Levy, Wex, & Bromme, 1999). Nevertheless, human CatC or CatX do not allow a clear classification into one of these three subfamilies.

Architecture, substrate specificity and catalytic mechanism: Cysteine cathepsins are monomeric enzymes except CatC that forms a tetramer (see paragraph: II.3 Structural characteristics and proteolytic specificity) (Turk, et al., 2001; Turk, et al., 2012). They are structurally based on the papain-like fold, which consists of two domains (left (L) and right (R) domains) of similar size with the V-shape active site cleft extending along the two-domain interface (McGrath, 1999; Turk, Turk, & Turk, 1997). The L domain encompasses three α-helices while the R domain is primarily built on a β-barrel. The active site Cys25 is located in the central ~30 residues long α-helix of the L-domain whereas His159 is positioned in the β-barrel of the R-domain on the opposite site of the active site

cleft (papain numbering). Cys25 and His159 form a thiolate-imidazolium ion pair (catalytic dyad) that is stabilized by Asn175 *via* a hydrogen bond (Storer & Menard, 1994). The nucleophilic cysteine residue has a remarkably low pKa value (pKa ~ 2.5-3.5) and is already ionized as a thiolate prior to substrate binding. During peptide hydrolysis, the nucleophilic Cys25 attacks the carbonyl carbon of the scissile bond of the bound substrate and forms a tetrahedral intermediate that is stabilized by an oxyanion hole. The tetrahedral intermediate converts into an acyl enzyme (enzyme-substrate thioester) with the simultaneous release of the C terminal portion of the substrate. This step is followed by the hydrolysis of the acyl enzyme with water (establishing a second tetrahedral intermediate), which finally divides into the free (i.e. unbound) enzyme and the released N-terminal portion of the substrate (deacylation step). Most of cysteine cathepsins are endopeptidases. Exceptions are CatB, which may act both as an endopeptidase and a carboxypeptidase and CatH, which is both an endopeptidase and an aminopeptidase; ultimately CatX and CatC are unequivocally exopeptidases (Lecaille, et al., 2002; Turk, et al., 2012). Mammalian cathepsins are optimally active and stable at a slightly acidic pH, and are rapidly inactivated at neutral or weakly basic pH, with the noticeable exception of CatS, which holds activity and enhanced stability at a neutral pH over other family members (Wilkinson, et al., 2015).

Localization and regulation: Cathepsins are synthesized as inactive preproenzymes. The signal peptide is removed during its translocation to the endoplasmic reticulum, where they are further post-translationally modified in the Golgi by N-glycosylation, which is crucial for engagement with mannose-6-phosphate receptors and transport to the endosomal/lysosomal compartments (Erickson, 1989; Linke, Herzog, & Brix, 2002). In the acidic organelles, procathepsins are processed to their active forms, either autocatalytically or in the presence of other proteases (e.g. aspartic CatD or legumain) (Dall & Brandstetter, 2016; Mach, Mort, & Glossl, 1994; Turk, et al., 2012). In contrast to other cysteine cathepsins, both CatC and CatX, because of their strict exopeptidase activity, cannot be autocatalytically activated (Dahl, et al., 2001; Hamon, Legowska, et al., 2016b). Despite the fact that the cathepsins are primarily found in acidic compartments, alternative locations were reported for these proteases. Independent mannose-6-phosphate transport has been reported, due to a defect of

mannose-6-phosphate receptors and/or the lack of mannose-6-phosphate signals, resulting in a constitutive secretion of proforms *via* classical secretory pathways (Brix, Dunkhorst, Mayer, & Jordans, 2008). Also cathepsins lacking the signal peptide were retrieved in the mitochondria or in the nucleus (Goulet, et al., 2004; Muntener, Zwicky, Csucs, Rohrer, & Baici, 2004; Tamhane, et al., 2016). Albeit their secretion may occur under normal physiological processes (e.g. bone remodeling, prohormone processing), the over-expression followed by a subsequent over secretion of cathepsins into the extracellular space is consistently a hallmark of pathophysiological settings (Fonovic & Turk, 2014a; Kramer, et al., 2017; Olson & Joyce, 2015; Reiser, et al., 2010). Additionally, extracellular CatB may bind to membrane caveolae and remains proteolytically active in tumor cells (Mohamed & Sloane, 2006). Cysteine cathepsins are controlled in various ways, namely at the transcriptional, translational, and post-translational levels. Regulatory sites have been found in the promoter region of some cathepsin genes (i.e. CatB, CatL, CatS or CatK) including Sp1 and Sp3 binding sites, or the interferon-stimulated response element (IRSE) (Lecaille, et al., 2008; Wilkinson, et al., 2015). At the protein level, cysteine cathepsins are synthesized as pre-proenzymes, with the presence of a proregion. This propeptide, which takes part in the proper folding, occludes the active site, rendering the protease inactive. Following the maturation of the zymogen to its active form, the released propeptide may still bind to and inhibit competitively its parent enzyme (Coulombe, et al., 1996; Groves, Coulombe, Jenkins, & Cygler, 1998; Wiederanders, Kaulmann, & Schilling, 2003). Moreover cathepsins are regulated by unspecific α2-macroglobulins that inhibit all classes of proteases, by specific protein inhibitors of the cystatin family (stefins, cystatins, kininogens; family I25, clan IH), but also by thyropins and some serpins (serine proteinase inhibitors) such as squamous cell carcinoma antigen 1, hurpin and endopin 2C (Abrahamson, Alvarez-Fernandez, & Nathanson, 2003; Barrett, 1986; Lenarcic & Bevec, 1998; Travis & Salvesen, 1983; Turk & Bode, 1991; Turk, Stoka, & Turk, 2008). Additional levels of control exist at the cellular stage. A primary level of control is conferred by compartmentalization, where cathepsins are normally confined to the endo-lysosomal lumen, which prevents uncontrolled proteolysis of host proteins and also stabilizes cathepsins in an acidic pH environment. Likewise the proteolytic activity of cathepsins may depend on Zn^{2+} and some metal ions, ionic strength, variations of redox potential, reactive oxygen and nitrogen species, and also

glycosaminoglycans (negatively charged mucopolysaccharides) (Fonovic & Turk, 2014a; Godat, et al., 2008; Jordans, et al., 2009; Lockwood, 2002, 2013; Percival, Ouellet, Campagnolo, Claveau, & Li, 1999; Sage, et al., 2013; Tatara, Suto, & Itoh, 2017) (**Figure 1**).

2.2 CatC biosynthesis, processing and maturation

In mammals, CatC is expressed mostly in the lung, spleen, kidney, liver and in myeloid cell lineages, in particular in neutrophils, mast cells, monocytes, macrophages and their precursors (Rao, Rao, & Hoidal, 1997). CatC is initially synthesized as a 55-kDa monomeric single chain pro-enzyme containing an "exclusion" domain (Asp1-Gly119), a propeptide (Thr120-His206), a heavy chain (Leu207-Arg370) and a light chain (Asp371-Leu439) (Dahl, et al., 2001) (**Figure 2A**). The heavy and light chains form a papain-like structure. After biosynthesis, the propeptide of 87 residues acts as an intramolecular chaperone for the folding and stabilization of pro-CatC into dimers. Although the stage where pro-CatC dimers associate to form tetramers has not been elucidated in cells, tetramers seem to be formed immediately after excision of the propeptide (Dahl, et al., 2001). Mature CatC purified from human kidney is an oligomeric enzyme that is functional as a tetramer (Dolenc, Turk, Pungercic, Ritonja, & Turk, 1995). In contrast to other cysteine cathepsins, pro-CatC is not matured by auto-catalytic processing. Dahl et al., reported that human pro-CatC expressed in baculovirus-transfected insect cells can be activated by CatL and S *in vitro*, a process initiated by the proteolytic activation through the removal of the propeptide (Dahl, et al., 2001). These proteases process pro-CatC *in vitro* in two consecutive steps. The first step results in the release of the exclusion domain and two peptides of 36 and 33 kDa, composed of the truncated propeptide, the heavy and light chains respectively. The second step corresponds to the release of the heavy chain from each peptide (Dahl, et al., 2001; Hamon, Legowska, et al., 2016b; Korkmaz, Lesner, et al., 2013). High-resolution X-ray diffraction analysis of human CatC showed that the heavy chain, the light chain and the exclusion domain are held together by non-covalent interactions (Turk, et al., 2001) (**Figure 2B**).

It has been shown however, that CatL and CatS are not required for pro-CatC activation in mice (Mallen-St Clair, et al., 2006). We have recently studied the maturation of pro-CatC in human neutrophilic precursor cell lines (PLB-980 and HL60). An almost complete inhibition of CatS by a cell

permeable chemical inhibitor was not sufficient to totally block the maturation of CatC, suggesting that other proteinase(s) may be involved in this process (Hamon, Legowska, et al., 2016a). We investigated pro-CatC maturation by various cysteine cathepsins sharing similar proteolytic activity and specificity to CatL and CatS and identified CatF, CatK and CatV as putative pro-CatC-activating candidates. Our results also suggest that pro-CatC maturation is a redundant process and that pro-CatC can be proteolytically activated by quite a few cathepsins, differing by their cell- or tissue-specific expression pattern *in vivo* (Lamort et al., unpublished).

The lysosomal localization of cathepsins in general and their ability to maturate pro-CatC *in vitro* is a strong indication for a role as primary activators of pro-CatC *in vivo*. CatF and CatL are widely expressed in human tissues suggesting that they are mainly involved in general lysosomal protein turnover and degradation. However, CatK, CatS and CatV are characterized by a more restricted cell and tissue-specific distribution, which suggests supplementary specialized functions. CatK is present at high levels in osteoclasts and plays an important role in bone resorption (Asagiri & Takayanagi, 2007; Novinec & Lenarcic, 2013). Human CatV is mainly expressed in the thymus and testis (Bromme, Li, Barnes, & Mehler, 1999), while CatS is predominantly found in the cells of the immune system. We assume that pro-CatC activation by cysteine cathepsins may vary in different tissues or cell types.

2.3 Structural determinants of proteolytic specificity and activity detection

2.3.1 Structural characteristics of substrate-binding sites

Studies of the unique structural and functional properties of active CatC were made possible by the development of a method for high level expression and purification of homogeneous active recombinant CatC and pro-CatC from baculovirus-infected insect cells (Dahl, et al., 2001; Lauritzen, et al., 1998), which subsequently enabled the elucidation of the structure of CatC (Molgaard, et al., 2007; Turk, et al., 2001). Mature CatC consists of four identical monomers with their active site clefts fully exposed to the solvent (Turk, et al., 2001) (**Figure 3**). The heavy chain of each monomer contains the catalytic Cys234 on the conserved central α-helix. The catalytic dyad is formed by Cys234 and His381. The available crystal structures of CatC in complex with peptide-based inhibitors

provide an insight into the substrate binding pockets (Furber, et al., 2014; Molgaard, et al., 2007). The exclusion domain unique to CatC is responsible for the aminopeptidase activity of this enzyme (Turk, et al., 2001). The carboxylic group of the Asp1 side chain that is conserved in all known CatC activation domain sequences, is responsible for the anchoring of the N-terminal amino group of CatC substrates (Molgaard, et al., 2007). The presence of the exclusion domain blocks the enzyme active site beyond the S2 subsite, making it only accessible to the N-terminus of its substrates (**Figure 4**). The CatC S2 subsite is the deepest site and it has the shape of a pocket. Its bottom is filled with a chloride ion and two solvent molecules (Turk, et al., 2001). The S1 site is located on the surface of the protease and is exposed to the solvent. Its large size allows for the accommodation of bulky and hydrophobic amino acid residues (Poreba, et al., 2014; Tran, Ellis, Kam, Hudig, & Powers, 2002). The S1' site is rather shallow. Beyond the S2' site, the active site cleft area is wide open, indicating that there is no particular site defined for the binding of substrate residues (Legowska, et al., 2016).

2.3.2 Substrate specificity

The substrate specificity of active CatC has been studied using peptides hormones (McDonald, Callahan, Zeitman, & Ellis, 1969; McDonald, Zeitman, & Ellis, 1972) and synthetic peptides as substrates (Poreba, et al., 2014; Tran, et al., 2002). CatC acts as dipeptidyl aminopeptidase with broad substrate specificity. CatC cleaves two-residue units until it reaches a stop sequence: neither can it accommodate an Ile at P1 position and a Lys, Arg or ornithine at P2 position, nor a Pro at P1 or P1' (Tran, et al., 2002). In any case, the protonation of the N-terminal amino group is required for the hydrolysis, as shown using dipeptide AMC substrates. The rather deep and narrow S2 subsite preferentially accommodates small and aliphatic amino acid residues such Ala and 2-aminobutyric acid (Abu) (Poreba, et al., 2014; Tran, et al., 2002). The carboxy group of Asp1 prevents accommodation of positively charged P2 side chains. The large size of S1 allows for the accommodation of bulky and hydrophobic amino acid residues (Poreba, et al., 2014; Tran, et al., 2002).

CatC exhibits optimal aminopeptidase activity in slightly acidic pH conditions and is activated by chloride ions at a pH below 7 (Cigic & Pain, 1999).

2.3.3 Proteolytic activity detection

Due to the narrow dipeptidylpeptidase activity of CatC, most of potent CatC substrates are synthetic dipeptide derivatives. The first reported substrates were Gly-Phe-p-nitroanilide (Gly-Phe-pNA) (Planta & Gruber, 1963) and Gly-Phe-β-naphthylamide (Gly-Phe-βNA) (Bury & Pennington, 1975). Over the years, Gly-Phe-7-amino-4-methylcoumarin (Gly-Phe-AMC) has become the most commonly used fluorogenic substrate. Indeed AMC-derived peptides are usually more sensitive than chromogenic substrates. Peptidyl AMCs were originally developed for chymotrypsin (Zimmerman, Yurewicz, & Patel, 1976) and were later used to characterize the broad specificity of CatC. Poreba et al., used fluorogenic dipeptide substrate libraries to compare the substrate specificity of three CatC orthologs from *Homo sapiens*, *Bos taurus*, and *Plasmodium falciparum* (Poreba, et al., 2014). The optimal substrates of human CatC contained small aliphatic residues (Gly, Ala, Abu, or Met) at the P2 position, and hydrophobic residues (phenylalanine, homophenylalanine (Hph), 4-benzoyl-phenylalanine (Bpa) or 6-benzyloxy-norleucine (Nle(6-*O*Bzl)) at the P1 position.

Selective and sensitive fluorescence resonance energy transfer peptides were recently developed to measure human CatC activity (Legowska, et al., 2016). Two series of tetra- and pentapeptide substrates were synthesized allowing to study the S′ specificity of CatC. The highly specific substrate Thi-Ala(Mca)-Ser-Gly-Tyr(3-NO_2)-NH_2 was selected for the detection of CatC activity in complex biological samples such as cell lysates, urine and bronchoalveolar lavage fluids (Legowska, et al., 2016).

In 2007, Li et al., reported a series of rhodamine (Rd) peptide substrates designed to monitor CatC activity in intact cells (J. Li, et al., 2009). The substrate (Abu-Hph)2-Rd allowed for sensitive and selective monitoring of CatC proteolytic activity in living cells by means of flow cytometry. Thong et al., developed a simple non-invasive fluorescence assay in fresh and cryopreserved cells of a human monocytic leukemia cell line by using the cell-permeable substrate Gly-Phe-7-Amino-4-trifluoromethylcoumarin (Gly-Phe-AFC).

Two different activity-based probes (ABP) for the detection of active CatC were also described. L-alanyl-4-iodo-L-phenylalanyl diazomethylketone (Ala-Phe(p-^{125}I)-CHN_2) was used to label CatC

activity in intact U937 cells (Methot, et al., 2007). In order to visualize CatC, autoradiography of proteins extracted from cell lysates was performed. In 2006, Yuan et al., reported FY01, a selective ABP for labeling of CatC in complex proteasomes, as well as in intact cells (Yuan, Verhelst, Blum, Coussens, & Bogyo, 2006). The probe was based on the dipeptide, L-norvalinyl-L-homophenylalanine, with an extended alkyl spacer, a vinyl sulfone reactive group and a BODIPY-TMR (BODIPY, borondipyrromethene; TMR, Tetramethylrhodamine) tag. It was proven to be cell permeable and selective by specific labeling of CatC in the human MDA-MB-231 breast cancer cell line.

2.4 Subcellular localization and secretion

CatC is ubiquitous and mainly located in the lysosomal/endosomal compartments of cells. It is found as active protease in the Golgi apparatus of granular-free undifferentiated human myelomonoblastic PLB-985 cells. Because it is co-localized with active NSPs in the Golgi, the activation process of granular proteases start probably in this compartment before the formation of intracellular granules. In promyelocytic HL60 precursor cells and *in vitro* differentiated neutrophil-like cells, active CatC is stored in cytoplasmic granules (Hamon, Legowska, et al., 2016b).

Pro-CatC is mainly secreted by constitutive secretion from the Golgi apparatus into the extracellular medium (Hamon, Legowska, et al., 2016b). Upon chemical or physiological stimuli active CatC is liberated into the extracellular milieu with other lysosomal enzymes by neutrophils, mast cells and lympocytes (Brown, McGuire, & Thiele, 1993; Hamon, Legowska, et al., 2016b; Wolters, Raymond, Blount, & Caughey, 1998).

Pro-CatC secreted by bronchial cells and resident alveolar macrophages is found in lung secretions from healthy individuals. However, CatC activity is detected in the lung secretions of patients suffering a chronic inflammatory lung diseases such as cystic fibrosis or asthma dominated by a neutrophilic inflammation (Hamon, Legowska, et al., 2016b; Legowska, et al., 2016). The enzyme concentration correlates with neutrophil numbers, which has also been experimentally confirmed in bronco-alveolar lavage fluid of macaques after lipopolysachharride-induced lung inflammation. The data support that active CatC can be used as a biomarker of active pulmonary neutrophilic

inflammation. The presence of active extracellular CatC has also been reported in other pathophysiological conditions including squamous carcinogenesis (Ruffell, et al., 2013) and central nervous system inflammation (Koike, et al., 2013)

Pro-CatC and mature CatC are constitutively found in the urine of normal subjects. Although the source of urinary CatC remains to be elucidated, it could be constitutively secreted by renal and/or bladder epithelial cells (Hamon, Legowska, Fergelot, et al., 2016).

3. CATC DEFICIENCY AND PLS

3.1 PLS

PLS (OMIM: 245000) is a rare condition with a Mendelian autosomal recessive inheritance, first described in 1924 (Papillon & Lefèvre, 1924). It is characterized by symmetric thickening of the palms and soles (palmo-plantar keratoderma, PPK), periodontitis and abscesses. It affects 1-4 people per million, males and females equally, (Gorlin, Sedano, & Anderson, 1964; Haneke, 1979) with parental consanguinity in 30-50% of reported cases. Haim-Munk syndrome (HMS) (OMIM: 245010) is a rarer and more severe phenotypic variant with additional features (Haim & Munk, 1965). PLS occurs worldwide, but most patients with HMS can trace their ancestry back to the original family who came from an inbred Jewish community, numbering around 2000 people, in Cochin, India; many migrated to Israel and Hart later documented the pedigree with 50 affected individuals, all of whom have been reported in various publications (Hart, et al., 1999). PLS and HMS are both caused by mutations in the CatC gene (Hart, et al., 1999; Hart, et al., 2000; Rai, et al., 2010) which encodes the lysosomal protease CatC (Ketterer, et al., 2017). It is likely that other factors contribute to the phenotype, since extreme differences in severity occur within families (Janjua, Iftikhar, Hussain, & Khachemoune, 2008) and conversely cases of HMS and PLS have been reported with the same homozygous mutation (Sulak, et al., 2016). Furthermore, in a series of 47 patients from 29 families in Saudi Arabia, there was no correlation between the severity of cutaneous and dental manifestations

using quantitative scoring systems (maximum scores 24 and 4 respectively) (Ullbro, Crossner, Nederfors, Alfadley, & Thestrup-Pedersen, 2003).

3.1.1 Skin manifestations

The skin appears normal at birth, but by 2-6 months scaling appears on the soles and later on the palms. Gradually the palmoplantar skin becomes thickened and red, particularly on the weight-bearing surfaces and painful fissures may occur. Later the plantar lesions may take on a punctate (Ullbro, et al., 2003), pitted or honeycomb-like (Moss, Spillane, Almquist, McCleskey, & Wisco, 2014) appearance. The keratoderma is transgredient meaning that it extends beyond the palmoplantar surfaces, involving the sides of the feet, dorsal surfaces of digits, malleoli and achilles tendons (**Figure 5A**). There may also be knuckle pads and sometimes there are discrete, red, scaly plaques over the knees and elbows, which can spread to involve the extensor aspects of the limbs, and regress leaving post-inflammatory hypopigmentation, a pattern strikingly similar to psoriasis. Rarely, there is generalized scaling or erythrokeratoderma (Janjua, et al., 2008). Of the 47 patients with PLS documented by Ullbro et al., 2 had ichthyosis and 3 had extensive psoriasiform plaques over scalp, trunk, and extremities (Ullbro, et al., 2003). The original HMS patients had particularly extensive skin changes, with a generalized ichthyosis (Haim & Munk, 1965), but further reports have included HMS patients with relatively mild skin changes (Sulak, et al., 2016) and PLS patients with extensive skin involvement (Kanthimathinathan, et al., 2013). Severity and extent of skin lesions can vary within families (Janjua, et al., 2008), and fluctuate over time (Kanthimathinathan, et al., 2013), sometimes with worse cracking in winter (Al-Khenaizan, 2002; Dalgic, Bukulmez, & Sari, 2011); there is usually no improvement with age (Ullbro, et al., 2003).

Skin biopsy is rarely required since the diagnosis is clinical. Histology is anyway non-specific, with epidermal thickening (acanthosis and hyperkeratosis), some retention of nuclei in the stratum corneum (parakeratosis) and a non-specific perivascular mixed inflammatory infiltrate.

The pathogenesis of the PPK in PLS remains unclear but abnormal proteolysis may disturb the process of epidermal cornification (Moss, et al., 2014).

Plantar hyperhidrosis and odour have been reported but are a non-specific finding in PPK regardless of cause. Malignant melanoma has been documented in four patients with PLS, three of whom were Japanese (Nakajima, et al., 2008). However, there appears to be a wider association of malignant melanoma with PPK in Japanese individuals, not specific to PLS, possibly reflecting the higher incidence of acral malignant melanoma in Japanese people.

An association of oculocutaneous albinism in two apparently unrelated patients with PLS was caused by mutations in two different genes, *CTSC* at and *TYR*, both located within a narrow chromosomal segment, 11q14.2–14.3. Adjacent markers were also shared suggesting that the two families, both consanguineous, had inherited the same chromosomal segment from a common ancestor. Both probands had a relative with the same two disorders, as well as a relative with only one of the two disorders, showing that recombination could occur between the two loci (Hewitt, et al., 2004) This adequately explains the association of these two conditions.

Nail dystrophy tends to correlate with skin severity and ranges from normal or minimal horizontal ridging in PLS to massive thickening with increased curvature resulting in a claw-like appearance (onychogryposis) in HMS.

Standard treatment of the PPK is with topical emollients and keratolytics, but in severe cases retinoids are highly effective (see below). Treatments may be combined, with retinoids used during the more troublesome winter months and topical therapy at other times (Al-Khenaizan, 2002).

Pyogenic infections, usually staphylococcal, occur more frequently than normal in people with PLS and HMS. This is often the first presentation to medical professionals, leading to investigation for immune deficiency before the diagnosis of PLS is recognised. The commonest manifestation is skin abscess which may be recurrent; breast abscess also occurs and has been reported in an otherwise healthy heterozygous carrier (Taibjee, Zhang, Chapple, Thakkar, & Moss, 2005), although carriers are not generally considered to be at increased risk of infection. Multiple chronic renal abscesses were reported in a 5 year old girl with PLS who also had insulin dependent diabetes: the organism isolated in that case was E.coli (Morgan, Hannon, & Lakhoo, 2011). Cerebral abscesses have occurred: multiple in a six year old boy (Kanthimathinathan, et al., 2013) and solitary in an adult woman (Pham, et al., 2004). However the most widely reported non-cutaneous pyogenic infection is liver abscess,

which may be single or multiple and can be fatal (Keskin-Yildirim, Simsek-Derelioglu, Kantarci, Yilmaz, & Buyukavci, 2012). While PLS is very rare, so is liver abscess in children: of 16 cases managed by pediatric surgeons over a 19 year period, 2 (12.5%) proved to be caused by PLS: thus PLS should be considered a relatively common cause of liver abscess in a child (Oguzkurt, Tanyel, Buyukpamukcu, & Hicsonmez, 1996). Bergman, re-investigating 5 members of the original Haim-Munk family after 20 years, found that most but not all affected individuals suffered repeated skin infections; furthermore one had died aged 12 years of intra-abdominal abscesses, one suffered perforation of a liver abscess into the lung and one developed tuberculoid leprosy treated effectively with dapsone (Bergman & Friedman-Birnbaum, 1988). Even when the diagnosis of PLS or HMS is known, physicians and surgeons treating the abscesses may not recognize a link with the underlying condition (Kanthimathinathan, et al., 2013). For this reason pyogenic infections in PLS and HMS are probably under-reported (Taibjee, et al., 2005).

Pyogenic infections in PLS and HMS are generally attributed to defective neutrophil killing of oral bacteria spread *via* the bloodstream. The disrupted epidermal surface of PPK may also harbour bacteria although the skin lesions do not appear clinically infected. Some patients with otherwise classical PLS do not appear prone to infections, perhaps because other anti-infective mechanisms can be deployed in the absence of CatC (Pham, et al., 2004).

3.1.2 Dental manifestations

PLS is characterised by aggressive periodontitis leading to premature loss of both deciduous and permanent teeth. The deciduous teeth erupt normally although microdontia, root resorption and incomplete root formation have been reported (Baghdady, 1982). From 2-3 years of age there is rapid destruction of the periodontal ligament with extensive bone resorption, creating deep periodontal pockets exuding pus. Symptoms include pain on eating and brushing, unpleasant odour and tender regional lymphadenopathy (Joshi, Dayal, & Kansagra, 1985). Examination reveals marked gingival inflammation with mild plaque accumulation. Plaque accumulation is generally associated with mobile teeth where discomfort prevents good oral hygiene. Caries is unusual due to the short life-span of teeth (Upadhyaya, Pfundheller, Islam, & Bhattacharyya, 2017). There is looseness, hypermobility, drifting,

migration, and exfoliation of teeth with roots intact. Normal serum alkaline phosphatase excludes hypophosphatasia as the cause (Bloch-Zupan, 2016) (**Figure 5B**).

The child may become completely edentulous, following which the gingiva returns to a healthy appearance only to relapse when the permanent dentition erupts. The permanent teeth are usually lost by age 14-15 years, again rendering the child edentulous (Papillon & Lefèvre, 1924) and allowing the gingival tissues to recover. Later erupting wisdom teeth may be spared (Glenwright & Rock, 1990).

Radiographic examination reveals generalised loss of alveolar bone (Dhanrajani, 2009) giving the appearance of teeth "floating in air". Resorption of both maxillary and mandibular ridges results in reduced facial height (Kola, 2014) (**Figure 5B**).

Several micro-organisms are implicated in the dental pathogenesis including *Aggregatibacter actinomycetemcomitans* (Albandar, Khattab, Monem, Barbuto, & Paster, 2012), *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Treponema denticola* (Saglie, Marfany, & Camargo, 1988; Stabholz, Taichman, & Soskolne, 1995; Tinanoff, Tempro, & Maderazo, 1995; Wara-aswapati, Lertsirivorakul, Nagasawa, Kawashima, & Ishikawa, 2001). Dental treatment aims to remove microorganisms, prevent further destruction of the periodontal ligament and allow healing (Dhanrajani, 2009). Appropriate conventional measures include scaling and root planing, oral hygiene instructions, 0.2% chlorhexidine gluconate mouth rinses and systemic antibiotics (Sreeramulu, Shyam, Ajay, & Suman, 2015). Ullbro et al., advocated proactive treatment for both primary and permanent dentition with strict oral hygiene measures, extraction of teeth affected by periodontal disease and removal of remaining primary teeth 6 months before the eruption of permanent teeth together with Amoxicillin 20-50 mg/kg/day or Amoxicillin + Clavulanic acid 20-40 mg/kg/day) (Ullbro, Brown, & Twetman, 2005). Permanent teeth with signs of periodontal disease should be treated by scaling with prophylactic antibiotic for 4 weeks (Amoxicillin 20-50 mg/kg/day + Metronidazole 15-35 mg/kg/day t.d.s.). Teeth where bone loss exceeds 30% of root length should be extracted. Unfortunately none of these measures is reliably effective (Bimstein, Lustmann, Sela, Neriah, & Soskolne, 1990; Bullon, et al., 2014; De Vree, Steenackers, & De Boever, 2000; Glenwright & Rock, 1990; Hathway, 1982; Hattab, Rawashdeh, Yassin, al-Momani, & al-Ubosi, 1995; Rateitschak-Pluss & Schroeder, 1984).

Systemic retinoids (etretinate and acitretin) may modulate the course of periodontitis and preserve teeth (Gelmetti, Nazzaro, Cerri, & Fracasso, 1989) but rarely halt progression. More effective treatment strategies require a better understanding of the underlying pathogenesis.

Early loss of teeth significantly affects children psychologically and socially; aesthetic management improves self-esteem which in turn impacts on academic performance (Kaur, et al., 2017). Rehabilitation comes in the form of dentures placed upon extraction of periodontally involved teeth, allowing patients time to adapt to the loss of teeth. Osseointegrated implants are contra indicated in growing individuals (Mankani, Chowdhary, Patil, Nagaraj, & Madalli, 2014) but implant-supported dentures with bone augmentation can improve long term oral rehabilitation in PLS (Kinaia, Hope, Zuhaili, & Tulasne, 2017).

3.1.3 Skeletal abnormalities and other features

HMS is differentiated from PLS by skeletal anomalies: tall, slim stature, pes planus and long, tapering fingers with distal translucency (osteolysis) on X-ray. Fixed contractures of digits and distal limbs (Janjua, et al., 2008) may result from severe skin involvement, but a rapidly destructive symmetrical arthritis of wrists and shoulders developed in one member of the original HMS family in her twenties (Lidar, Zlotogorski, Langevitz, Tweezer-Zaks, & Zandman-Goddard, 2004); wrist Xrays showed joint-space narrowing and subchondral cysts, MRI of the shoulder revealed hypertrophied synovium, synovial histology showed hyperplasia with a plasma cell infiltrate and further investigations showed no other underlying disease. The authors noted that synovial epithelial shares some characteristics with periodontal junctional epithelium. CatC is strongly expressed in osteoclasts that may also be relevant to the skeletal manifestations (Hart, et al., 2000).

A 1979 review of 150 published cases found that affected individuals had no other consistent abnormalities and carriers had no definite manifestations (Haneke, 1979). Mild intellectual disability was reported in 7 cases but this was probably coincidental. Ectopic calcification of the dura mater, falx cerebri, tentorium cerebelli and choroid plexus are seen radiologically (Gorlin, et al., 1964) but with no apparent clinical consequences. E. Haneke found 7 reports of intracranial calcification, with no neurological deficit recorded in those cases (Haneke, 1979).

3.1.4 Treatment with retinoids

The retinoid drugs, vitamin A derivatives, are highly effective in a variety of hyperproliferative skin conditions, but their usage is limited by teratogenicity and other adverse effects. Acitretin, which has now replaced etretinate, is used in hyperkeratotic disorders such as psoriasis and ichthyosis. Isotretinoin, widely used for severe acne, is less effective in the hyperkeratotic conditions but may be preferred in women of child-bearing years because it clears more quickly from the body (1 month for isotretinoin, compared with 3 years for acitretin). There are numerous case reports showing excellent efficacy of etretinate and acitretin (usual dose 0.5-1.0 mg/kg) for the PPK of PLS: maximum improvement occurs by 4-6 weeks (Bergman & Friedman-Birnbaum, 1988), with relapse following cessation of treatment. Isotretinoin 0.5-1.0 mg/kg/day used in two young women was also highly effective (Nguyen, Greer, Fisher, & Cooper, 1986).

One author also reported a complete remission of pyogenic infections in 4 individuals descended from the original Haim-Munk family who were treated with etretinate over 21 months (Bergman & Friedman-Birnbaum, 1988) but spontaneous remissions of infections also occur. There is no evidence that retinoids increase the risk of liver abscess (Khandpur & Reddy, 2001).

Improvement of periodontitis with retinoids has been claimed (Lee, Wong, & Fischer, 2005; Nazzaro, Blanchet-Bardon, Mimoz, Revuz, & Puissant, 1988) but not proven (Al-Khenaizan, 2002), even if commenced after clearance of the primary dentition and prior to the eruption of the permanent dentition.

The mechanism of action of retinoids on PPK is unclear: it may be a simple antiproliferative action since acitretin is often effective in other types of PPK. However, retinoids also have effects on the immune system, including regulation of CatC expression (Sanchez-Martinez, et al., 2014), which might be relevant in PLS.

3.2 *CTSC* and the genetics of PLS

The mutated CatC gene (*CTSC)* in PLS was mapped to chromosome bands 11q14-q21 on the long arm of chromosome 11 (**Figure 6**) using a traditional linkage approach and/or homozygosity mapping (identifying areas of shared homologous chromosomal regions) by Laass and colleagues

(Laass, et al., 1997) and Fischer and colleagues in 1997 (Fischer, et al., 1997). The location was further refined by Hart et al., (Hart, et al., 1998). All these studies revealed a lack of genetic heterogeneity in PLS, with the condition exhibiting linkage to the same genetic region in all families studied.

Toomes et al., narrowed the region harbouring the gene further to a 1.2 cM interval by homozygosity mapping and combing data with those from the previous study (Toomes, et al., 1999). This region harboured several genes including *CTSC* at chromosome band 11q14.2. This was selected as the candidate gene because some lysosomal disorders such as Chediak- Higashi syndrome are associated with severe early-onset periodontitis. The structure of the gene was determined and loss-of-function mutations identified in all the families studied. In addition, functional analyses revealed almost complete loss of activity in affected individuals and reduced activity in carriers. Hart et al., also demonstrated mutations in *CTSC* in additional families (Hart, et al., 1999).

The CatC gene (DNA seq NC_000011.10) consists of 7 exons encoding multiple transcripts. At least one of the transcripts (mRNA seq NM_0018114.5) encodes a pre-pro-protein (protein seq NP_001805.3). Although PLS is genetically homogenous i.e., all individuals with PLS have mutations of same gene, *CTSC*, like many loss-of-function phenotypes, there is considerable allelic heterogeneity i.e., the pathological mutations are highly varied (Hewitt, et al., 2004; Nagy, et al., 2014; Selvaraju, et al., 2003). Pathological missense mutations constitute roughly half of all mutations identified (Nagy, et al., 2014) and have been shown to target critical sites in the mature protein (Hewitt, et al., 2004). These include not just mutations affecting the active sites in the enzyme but also mutations that impact the exclusion domain or the folding or aggregation of the CatC tetramer. A majority of the pathological missense mutations occur in the regions coding for the heavy chain (exons 5-7). In contrast, as might be expected, the nonsense mutations and frameshift mutations are more evenly dispersed (Nagy, et al., 2014).

In a large percentage of families (68%) with PLS, the affected individuals are homozygous for the mutations; in the remaining 32% of the families, affected individuals are compound heterozygotes i.e., have two different pathological mutations (Nagy, et al., 2014). Thus, in approximately two-thirds of the families, the parents of affected individuals are consanguineous and in a third of the families,

the parents of affected individuals are unrelated. The much greater incidence in children from consanguineous mating compared to children from non-consanguineous mating is consistent with the relatively low carrier frequency of PLS mutations in the population.

The pathological mutations result in an almost complete loss of CatC activity (Toomes, et al., 1999). Analyses in one unusual family suggested that levels as low as 13% may be sufficient to rescue normal phenotype (Hewitt, et al., 2004).

Affected individuals with pre-pubertal periodontitis have a similar early-onset destructive periodontitis as PLS, but lack the skin changes seen in PLS. The condition as originally described by Page and colleagues (Page, et al., 1983) was not necessarily inherited and comprised phenotypes that affected few teeth selectively or was more generalised. However, there are rare families with an autosomal recessive mode of inheritance of the condition. Hart et al, 2000 demonstrated *CTSC* mutations in a consanguineous family with non-syndromic pre-pubertal periodontitis and suggested that this condition is allelic to PLS (Hart, et al., 2000). This was confirmed by Hewitt et al., who demonstrated *CTSC* mutations in one of two further pre-pubertal periodontitis cases (Hewitt, et al., 2004). The mutations identified in pre-pubertal periodontitis do not form a separate class of mutations to those occurring in PLS. The same mutations are seen in both conditions and result in almost complete loss of CatC activity (Hewitt, et al., 2004). This suggests that the phenotype of PLS is variable and may present as pre-pubertal periodontitis without the skin changes. This is further supported by the demonstration of loss-of-function CatC mutations in patients with early-onset destructive periodontitis and mild skin changes (Noack, et al., 2008). The lack of *CTSC* mutations in affected individuals with pre-pubertal periodontitis in a consanguineous family and lack of a shared haplotype at the *CTSC* locus in affected individuals (Hewitt, et al., 2004) suggested that pre-pubertal periodontitis is a genetically heterogeneous condition. This was confirmed by (Noack, et al., 2008), who did not identify any *CTSC* mutations in two further families with pre-pubertal periodontitis.

Aggressive periodontitis is characterised by 3 main features: patients free of systemic predisposing conditions, rapid attachment loss and periodontitis, and familial aggregation (Albandar, 2014). The onset is circumpubertal, or in adolescence or early adult life. The condition can be generalised or localised but does not exhibit the rapidly progressing destructive periodontitis seen in

PLS. Hewitt et al., 2004 analysed CatC activity in this condition and found no difference in the activity between cases and controls (Hewitt, et al., 2004). This is not entirely unexpected, given that carriers of *CTSC* mutations with one normal copy of the gene and one mutated copy of the gene have not been reported to show any increased predisposition to periodontitis, and that almost complete loss of CatC activity is required to precipitate the PLS phenotype.

3.3 Neutrophils dysfunction in PLS

3.3.1 Role of neutrophils

Neutrophils are the dominant inflammatory-immune cell of humans accounting for 40-60% of all circulating leukocytes and bridging the innate and acquired immune systems as the major defense cell against microbial challenge within the periodontal tissues. Recruitment of neutrophils from the circulation into the periodontal tissues is initiated by pathogenic bacteria and their products (e.g. bacterial cell-wall components such as lipopolysaccharides), which permeate the tissues, serving as chemoattractants for host immune cells. Upon binding to host cell surface receptors, the bacterial ligands initiate a cascade of inflammatory-immune events, which includes the production of pro-inflammatory cytokines and additional chemoattractants (chemokines) that co-ordinate the recruitment of neutrophils and other immune cells to the site of infection *via* a chemical gradient-driven process known as chemotaxis.

At sites of infection or bacterial challenge, neutrophils employ a diverse repertoire of antimicrobial defense mechanisms to eradicate pathogens, which includes phagocytosis, a process of pathogen internalisation with subsequent destruction through the formation of reactive oxygen species (ROS) and exposure to activated lysosomal enzymes within the cell. ROS generation requires the co-ordinated assembly of an enzyme complex known as the NADPH-oxidase at the membrane in which the pathogen is contained (the phagosome) following activation of second messengers and activation of protein kinase C (PKC). Fusion of the phagosome with cytoplasmic granules/lysosomes enables entry of antimicrobial peptides into the phagosome, their activation by ROS and subsequent enzymatic degradation of the pathogenic bacteria. NSPs are contained within the azurophilic granules. Four NSPs have been characterized to date, NE, CatG, PR3 and NSP4 (de Haar, Hiemstra, van Steenbergen,

Everts, & Beertsen, 2006; Perera, et al., 2012). NSPs are known to be important in neutrophil killing of Gram-negative and Gram-positive bacteria, several of which are known periodontal pathogens (Belaaouaj, Kim, & Shapiro, 2000; Belaaouaj, et al., 1998; de Haar, et al., 2006; Reeves, et al., 2002). In addition to their role in phagocytosis, NSPs can be released extracellularly and can target various other host-derived cytokines, chemokines, growth factors and cell surface receptors. For example, PR3 can cleave the cytokine and neutrophil chemokine interleukin-8 (CXCL8) increasing its potency (Padrines, Wolf, Walz, & Baggiolini, 1994), in addition to activating the cell surface-bound pro-inflammatory cytokine TNFα (Armstrong, Godinho, Uppington, Whittington, & Millar, 2009).

Another neutrophil-mediated antimicrobial strategy involves the formation of neutrophil extracellular traps (NETs), which are composed of de-condensed nuclear chromatin/DNA structures decorated by various antimicrobial proteins, including NSPs (Brinkmann, et al., 2004). NETs facilitate the containment and subsequent destruction of invading microorganisms. In addition to their role as structural components of NETs, NSPs are also required for NET release (Neumann, et al., 2014; Papayannopoulos, Metzler, Hakkim, & Zychlinsky, 2010). Three methods of NET formation have been described:

1) Suicidal NETosis, which is ROS-dependent and can take up to 4 hours for NETs to be released into the extracellular milieu (Brinkmann & Zychlinsky, 2007; Neeli, Dwivedi, Khan, & Radic, 2009), a process requiring the activation of receptors such as toll-like receptor 4 (TLR-4) (Al-Khafaji, et al., 2016),

2) Vital NETosis, a more rapid pathway (up to 60 minutes) that appears to occur independently of ROS generation (Douda, Khan, Grasemann, & Palaniyar, 2015; Pilsczek, et al., 2010; Yipp & Kubes, 2013) *via* TLR and complement receptor (C3a) binding (Byrd, O'Brien, Johnson, Lavigne, & Reichner, 2013; Yipp, et al., 2012),

3) Vital NETosis involving the very rapid (several minutes) release of mitochondrial rather than nuclear DNA *via* C5a and lipopolysachharride binding (Yousefi, Mihalache, Kozlowski, Schmid, & Simon, 2009).

3.3.2 Consequences of neutrophil CatC deficiency in PLS

NSPs activation: Biosynthesis of NSP zymogens occurs normally in PLS patients with their localization detectable within the granules of immature neutrophils (Sorensen, et al., 2014). However, they are absent in mature PLS neutrophils (Perera, et al., 2013; Pham, et al., 2004; Sorensen, et al., 2014) (**Figure 7**), indicating that CatC inactivation promotes zymogen elimination during neutrophil maturation. This has been confirmed in PLS patients, whose neutrophils are characterized by low-to-a complete absence of NSP proteolytic activity, and very low protein levels in mature neutrophils within the circulation (Pham, et al., 2004; H. Roberts, et al., 2016). An autophagic dysfunction could explain the elimination of several proteins in PLS neutrophils including NSP zymogens (Bullon, et al., 2018). The NSP deficiency is believed to underlie the devastating severe periodontal disease in PLS patients; however, the rare nature of the disease has limited the systematic evaluation of neutrophil function in PLS, with many reports being restricted to analyses of neutrophils from individual patients.

NETosis: Several studies have reported on the absence of NSPs in PLS and the subsequent inability of neutrophils derived from patients to form NET structures (Eick, et al., 2014; H. Roberts, et al., 2016; Sorensen, et al., 2014). NE is known to be critical for NET formation as demonstrated in hereditary neutropenia, a condition characterized by a decreased number of circulating neutrophils (M. Horwitz, Benson, Person, Aprikyan, & Dale, 1999; M. S. Horwitz, et al., 2007) and an inability to produce NETs. It appears that a NETosis defect is stimulus-dependent in some PLS patients. For example, PLS neutrophils exposed to a ROS-dependent neutrophil stimulus (i.e. Suicidal NETosis), such as phorbol 12-myristate 13-acetate (PMA) cannot produce NETs (Sorensen, et al., 2014), however when stimulated with a ROS-independent inducer (i.e. Vital NETosis), such as calcium ionophore or bacteria such as Mycobacterium bovis, NET structures can form in these patients (Batinica, et al., 2017).

ROS production: Previous studies have demonstrated that PLS neutrophils produce enhanced levels of ROS following FcγR stimulation, or receptor-independent stimulation using phorbol 12-myristate 13-acetate (PMA) (Battino, et al., 2001; H. Roberts, et al., 2016). Increased ROS production

is associated with increased oxidative stress, ultimately contributing to collateral host tissue damage and compromised antioxidant micronutrient levels (Perera, et al., 2013). Increased ROS production in neutrophils has also been demonstrated in chronic periodontitis, in which neutrophils have been shown to exhibit enhanced ROS production both in the absence (Matthews, Wright, Roberts, Cooper, & Chapple, 2007) and presence (Matthews, Wright, Roberts, Ling-Mountford, et al., 2007) of bacterial stimuli, termed neutrophil hyper-activity and hyper-reactivity, respectively.

Cytokine release: Elevated levels of pro-inflammatory cytokine release has been reported from peripheral blood neutrophils derived from PLS patients, both in the absence and presence of bacterial stimuli (H. Roberts, et al., 2016). Other immune-regulation proteins have also been reported to be elevated in PLS neutrophils, including the S100A8 and S100A9 proteins, both of which are biomarkers of neutrophil activation (Foell, Wittkowski, Vogl, & Roth, 2007), and known targets of CatG (Ryckman, Vandal, Rouleau, Talbot, & Tessier, 2003). CatG acts on S100A9 to generate neutrophil immobilizing factor (NIF), which is involved in the inhibition of neutrophil chemotaxis (Goetzl & Austen, 1972), serving to limit neutrophil influx into inflamed tissues and therefore aiding the process of inflammation resolution. Increased systemic levels of the S100A8/A9 complex have been reported from PLS neutrophils, and, with a role in the formation of the NADPH-oxidase, this may account for the increased ROS activity characteristic of PLS neutrophils (Kerkhoff, et al., 2005). Increased circulating levels of cytokines have also been demonstrated in blood plasma from PLS patients (H. Roberts, et al., 2016), which may prime peripheral blood neutrophils prior to their exit from the circulation (Dias, et al., 2011), and in turn result in enhanced ROS activity within the oral tissues when pathogenic bacteria or their products are present at periodontal sites (Yao, et al., 2015).

Chemotaxis: Defective chemotaxis has been demonstrated in PLS patient neutrophils (H. Roberts, et al., 2016). A consequence of this is may be the inefficient movement of neutrophils through infected tissues. Such increased tissue transit times, alongside exaggerated ROS and cytokine release likely lead to enhanced host tissue destruction, as bystander damage in and around sites of infection. NSPs are known to de-activate specific neutrophil chemoattractants such as CXCL8 and macrophage

inhibitory protein-1 alpha (MIP1α), thus loss of NSP activity due to CatC deficiency, may disrupt vital stop-signals involved in neutrophil recruitment to infected tissue sites. *Ex vivo* studies of neutrophils from healthy individuals have demonstrated their inability to undergo cytoskeletal reorganisation if they lack the NSPs NE and CatG; a result of decreased phosphorylation of the GTPase RAC1, which is performed by activated CatG (Djawari, 1978).

3.3.3 Contribution of neutrophils to periodontal tissue destruction in PLS

For many years, the underlying cause of periodontitis in PLS patients was deemed largely due to failure of the hosts' antimicrobial peptide systems such as LL37, NE, CatG and the NET-complexed proteases, secondary to CatC deficiency, to eliminate pathogenic bacteria. For example, LL37, which is formed by the cleavage of the neutrophil specific granule precursor human cathelicidin-18 (hCAP18) by PR3 (Papayannopoulos, et al., 2010) has been shown to be important in NET formation. However, comprehensive evaluation of the impact of CatC deficiency upon NSP activity and neutrophil function has revealed a far more complex and inter-linked series of events, which implicate neutrophil dysfunction in the tissue destruction observed at sites of bacterial challenge, such as the periodontium (Perera, et al., 2013). The severe clinical inflammation and associated bone destruction that characterizes the pre-pubertal periodontitis in PLS appears consistent with this hypothesis. Historically, neutrophils were regarded as terminally differentiated, committed and short-lived killer cells, however, recent studies have revealed a life expectancy of 5.4 days within the circulation (Pillay, et al., 2010) and a capability to coordinate and orchestrate innate and acquired immune responses (Uriarte, Edmisson, & Jimenez-Flores, 2016), *via* cell-cell interactions and the release of signaling molecules (Ling, Chapple, & Matthews, 2015). Importantly, neutrophils from PLS patients do not exhibit a general defect in their ability to destroy bacteria (Pham & Ley, 1999; Sorensen, et al., 2014). For example they are able to kill bacteria *via* the generation of ROS, hypochlorous acid and by releasing antimicrobial peptides (Perera, et al., 2012; Williams, 2006), it seems that the defects manifest in PLS neutrophils arise from a chronic exposure to a microbial stimulus, such as around the teeth, or in a chronic skin infection.

NSPs possess a diverse array of activities, some pro-inflammatory and others inflammation resolving in nature (Pham, 2008). For example, NSPs have been shown to inactivate IL-6 at sites of inflammation in patients with acute inflammatory diseases (Bank, Kupper, & Ansorge, 2000), thereby exhibiting a role in orchestrating immune response signals. The NSPs NE and CatG are known to kill periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* (Bangalore, Travis, Onunka, Pohl, & Shafer, 1990), thus their deficiency in PLS likely compromises neutrophil antimicrobial capacity, allowing for persistence of specific bacterial pathogens and their continued stimulation of ineffective immune-inflammatory responses within periodontal tissues. This may explain the localization of areas of the tissue destruction in PLS to areas where neutrophils are the dominant immune cell. Besides periodontal disease, PLS patients suffer from dermatological disorders which are characterized by extensive neutrophil infiltration with cells reported to play a dominant pro-inflammatory role based upon histopathological examination of affected tissues (Naik & Cowen, 2013). Interestingly, the auto-inflammatory skin disease psoriasis is characterized by a high neutrophil presence in addition to dysregulated NET production and NSP activity (Majewski, et al., 2016).

Given the divers nature of NSP activity in the human inflammatory-immune system, there are two scenarios which appear likely to underpin the aggressive periodontitis in PLS, which are summarized in **Figure 8**.

1) Relentless recruitment of primed and/or activated neutrophils to the oral tissues. Studies on CXCL8 and MIP1α, both potent neutrophil chemoattractants, reveal them to be NSP targets for inactivation (Leavell, Peterson, & Gross, 1997; Ryu, et al., 2005). Interestingly, MIP1α is also an osteoclast activating factor, involved in bone resorption (Choi, et al., 2000). This provides insight into a subtler role for neutrophils in co-ordinating immune responses, as NSPs play an important role in regulating further neutrophil recruitment by silencing the inducers of neutrophil accumulation. Thus, in the absence of NSP activity, MIP1α may drive the relentless recruitment of neutrophils to the inflamed periodontal tissues; an area of the body under constant exposure to bacteria and their products as immune-stimulants. In the most common and less destructive form of periodontitis, chronic periodontitis, similar changes in neutrophil behavior compared to healthy controls have been

reported. These include similar trends in basal and stimulated neutrophil chemotaxis, ROS production and pro-inflammatory cytokine release, which contribute to a chronic non-resolving inflammatory state (Ling, et al., 2015; Matthews, Wright, Roberts, Cooper, et al., 2007; Matthews, Wright, Roberts, Ling-Mountford, et al., 2007; H. M. Roberts, et al., 2015). Moreover, other disorders in which NSP deficiency/dysfunction are characteristic, are also associated with an increased severity of periodontitis, including Chédiak-Higashi syndrome (Holt, Gallo, & Griffiths, 2006), Haim-Munk syndrome (Hart, et al., 2000) and specific granule deficiency (Gallin, et al., 1982).

2) The second scenario is likely due to compromised microbial killing by neutrophils in PLS. Deficient bacterial killing results in persistence of pathogenic species and rather than those pathogens directly causing tissue destruction, their presence is more likely to trigger indirect tissue damage mediated by the neutrophil dysfunction. The relentless recruitment of neutrophils by the persistent pathogenic stimulus, which histologically characterize the local periodontal inflammation, leads to excess ROS and pro-inflammatory cytokine release, which in turn drives connective tissue damage and bone loss. Interestingly, although reported to suffer only modest systemic infections, nearly 1/5th of all PLS patients suffer recurrent infections, mostly skin abscesses, but significant complications may result, including cerebral abscesses (Kanthimathinathan, et al., 2013).

Further support for a directly destructive role of neutrophils in PLS arises from other neutrophil disorders that involve an inability to generate ROS. In chronic granulomatous disease, there is a failure to assemble the ROS-generating NADPH oxidase complex. Whilst such patients suffer frequent systemic infections, there is little evidence for them exhibiting an increased risk of periodontal disease (Nussbaum & Shapira, 2011), thus hypo-function of neutrophil cannot, in itself be the sole explanation for the aggressive periodontitis characteristic of PLS; NSP function does not constitute the primary antimicrobial defense mechanism. It appears therefore, that the cause of periodontitis in PLS is likely a manifestation of multiple co-incident downstream effects of the CatC deficiency, upon NSPs involved in mediating immune responses to microbial challenge. An example of reduced killing in PLS the LL37 deficiency, which acts as a potent antimicrobial agent targeting the periodontal pathogens *Aggregatibacter actinomycetemcomitans* (D. Tanaka, Miyasaki, & Lehrer, 2000) and *Porphyromonas*

gingivalis (Greer, Zenobia, & Darveau, 2013). Furthermore the absence of NSPs, and therefore LL37, correlates with periodontal disease severity (Eick, et al., 2014), thus a lack antimicrobials supports a reduced capacity of neutrophils to eliminate invading agents both directly and indirectly.

In summary, the pre-pubertal periodontitis that characterizes PLS appears to result directly from *CTSC* mutations and provides valuable insights into the diverse, complex and important physiological roles played by CatC in this monogenetic disease The impairments in neutrophil function, resulting from the lack of NSPs appears to reduce bacterial clearance during infection and creates a destructive pro-inflammatory environment within the periodontal tissues characterized by increased ROS and cytokine generation, failure to inactivate CXCL8 and MIP1α, and leading to subsequent tissue destruction and alveolar bone loss. The vital role for NSPs in orchestrating a finely balanced immune homeostasis and regulate effective immune processes is evident from studies of PLS patients. Understanding the critical role of NSPs, may lead to therapeutic strategies to restore the protective functions of these enzymes in order to prevent the devastating periodontal tooth loss during adolescence that characterizes PLS.

4. BIOLOGICAL FUNCTIONS OF CATC

4.1 Maturation of neutrophil serine proteases (NSPs)

Intracellular proteolytic activities in leukocytes were discovered about 100 years ago and were already attributed to a “leukoprotease” postulated by Opie (Opie, 1922). The proposal that a deranged homeostasis between neutrophilic leukocyte proteases and their inhibitors contributes to chronic inflammatory lung disease has its roots in the 1960s (Janoff, 1985) and became and remained very popular as the protease-antiprotease imbalance hypothesis ever since for many researchers. In the 1970s, the major serine proteases of leukocytes were characterized and explored at the functional biochemical level. Later, with the advent of molecular cloning techniques, their complete structure and genetics were unraveled (Jenne, 1994). Whereas the first three members of NSPs, elastase (Okano, et

al., 1987) (using the synonym medullasin), CatG (Salvesen, et al., 1987) and PR3 (Jenne, Tschopp, Ludemann, Utecht, & Gross, 1990), were already cloned in the 1980s, the fourth member of NSPs, called NSP4, was only recently reported in (Perera, et al., 2012). Immune defense cells, including neutrophils, mast cells, lymphocytes and macrophages, express a unique subset of eleven single domain serine proteases, which all belong to one specific subclass. While a great majority of serine proteases cleaves after arginine and lysine residues, the highest diversification of cleavage specificities emerged in cell lineages of the immune system. NE, PR3 and CatG cleave after small aliphatic and aromatic residues (Korkmaz, et al., 2010; Korkmaz, et al., 2016; Korkmaz, Moreau, & Gauthier, 2008), while NSP4 cleaves selectively after arginine, but not lysine residues (Perera, et al., 2013).

NSPs evolved from a common ancestor through gene duplication and belong to the trypsin/chymotrypsin superfamily of serine proteases. Their genes consist of five exons and four introns (Caughey, et al., 1993; Zimmer, et al., 1992). Single genes encoding for NE and PR3, called *ELAN*E and *PRTN3*, respectively, are located in the same cluster on chromosome 19p13.3. The gene encoding for CatG, *CTSG*, is located on chromosome 14q11.2 in a cluster containing the genes for chymase, granzyme B (Caughey, et al., 1993) and granzyme H (Fellows, Gil-Parrado, Jenne, & Kurschus, 2007).

During the early stages of neutrophil maturation (myeloblast/promyelocyte stages) (Garwicz, et al., 1997; Garwicz, Lindmark, Persson, & Gullberg, 1998; Gullberg, Andersson, Garwicz, Lindmark, & Olsson, 1997; Rao, Rao, Marshall, & Hoidal, 1996) NSPs are transiently synthesized as inactive zymogens with a dipeptide at the amino-terminus and a longer C-terminal extension. The NSP zymogen structure prevents premature proteolysis in the endoplasmatic reticulum which would be cytotoxic for host cells (Jenne & Kuhl, 2006). Activation of NSPs by CatC occurs during intracellular transport and packaging in acidic primary granules and involves the removal of the N-terminal dipeptide to allow the active site to become accessible to substrates. The observation by Adikson et al. that all NSP activities in neutrophil lysates from CatC-deficient mice were severely reduced revealed the important dominant role of CatC in the activation of NSPs *in vivo* (Adkison, Raptis, Kelley, & Pham, 2002). CatC is the major but not the only protease capable of converting NSP zymogens into active proteases during biosynthesis and transport into granules (Seren et al., unpublished).

NSPs display a 3D structure consisting of two homologous β-barrel domains and a C-terminal α-helix (Bode, et al., 1986; Fujinaga, Chernaia, Halenbeck, Koths, & James, 1996; Hof, et al., 1996). The highly conserved residues of the catalytic triad (His57, Asp102, Ser195; chymotrypsin numbering) are located at the interface between the two β-barrels. The removal of the N-terminal dipropeptide by CatC during activation (**Table 3**) results in a re-orientation and remodeling of the activation domain surface loops (174-loop, 217-225-loop, 180-loop and autolysis loop) of the protein (Korkmaz, et al., 2016). The formation of an internal salt bridge between the free ammonium group of Ile16, the first N-terminal residue, and the side-chain carboxylate of Asp194 renders the S1 pocket of the active site accessible to the substrates. Resolved X-ray structures of pro- and mature forms of related serine proteases illustrated the topological differences before and after activation (Hink-Schauer, et al., 2002; Jenne & Kuhl, 2006; Reiling, et al., 2003). The crystal-based structure of human pro-chymase suggests that the mobile, "dangling" dipropeptide on the zymogen surface increases accessibility to CatC, and likely improves cleavability (Reiling, et al., 2003). After removal, the neo-amino-terminus of the mature serine protease dives into the interior of the globular protein (Pereira, et al., 1999), where it is protected from further processing (**Figure 9**).

NSPs contain an important number of positively charged residues in their structures and thus are highly cationic proteases (Korkmaz, et al., 2007; Korkmaz, Moreau, et al., 2008). A positively charged cluster is located on the activation domain of both human NE and PR3. However, this positive cluster is disorganized by negative residues on human PR3 (Korkmaz, et al., 2007). A surface hydrophobic patch constructed of five hydrophobic residues is present on human PR3 (Korkmaz, Kuhl, Bayat, Santoso, & Jenne, 2008). Sequence alignments of PR3 from various rodents and primates revealed that this hydrophobic patch was only conserved in the PR3 from chimpanzee (Korkmaz, Kuhl, et al., 2008; Kuhl, et al., 2010). It confers PR3 with the unique property to be present at the cell surface of quiescent circulating neutrophils (Csernok, Ludemann, Gross, & Bainton, 1990; Halbwachs-Mecarelli, Bessou, Lesavre, Lopez, & Witko-Sarsat, 1995). All NSPs however are liberated into the extracellular medium in response to neutrophil activation, with a small fraction that remains exposed on the cell surface. NE and CatG are expressed on the neutrophil cell surface by charge-dependent mechanisms

(Campbell & Owen, 2007). Membrane-bound PR3 (mPR3) is released from activated neutrophil cell surfaces in macrovesicles (Hong, et al., 2012), which may promote systemic inflammation in disease conditions (Martin, et al., 2016).

The proteolytic activity of NSPs is controlled by the endogenous protein inhibitors belonging to the family of serpins (e.g. α1-antitrypsin (AAT), α1-antichymotrypsin, monocyte neutrophil elastase inhibitor) and chelonianin of canonical inhibitors (secretory leukocyte protease inhibitor, elafin) (Korkmaz, et al., 2010; Korkmaz, et al., 2016; Korkmaz, Moreau, et al., 2008). Serpins (serine proteinase inhibitors) are irreversible inhibitors of serine proteases that include more than 1000 monomeric proteins found in both the animal kingdom, the plant kingdom and viruses (Olson & Gettins, 2011; Potempa, Korzus, & Travis, 1994; Silverman, et al., 2001). Most serpins consist of 350 to 500 residues, whose inhibitory reactive site is located within an exposed and mobile loop comprising about twenty residues. Serpins interact with their target proteases according to a unique mechanism: the interaction of a serpin with the target protease allows the formation of an initial non-covalent reversible complex (Gettins, 2002). The cleavage of the reactive loop of a serpin by the protease between P1-P'1 bond leads to the covalent complex which evolves either towards a complex where the protease is complexed to the serpin (inhibitory pathway), or towards the release of the irreversibly inactivated cleaved serpin while the protease is being regenerated (substrate pathway) (Olson & Gettins, 2011). Canonical inhibitors include different families with different folding modes. Nevertheless, they all have in common an inhibitory loop exposed to the solvent, of variable sequence but with a very similar conformation in the P3-P'3 region, thus called canonical conformation (Bode & Huber, 1992). Virtually all canonical inhibitors respond to the minimum reaction scheme called the standard mechanism (Krowarsch, Cierpicki, Jelen, & Otlewski, 2003; Laskowski & Kato, 1980). The reversible complex between protease and inhibitor is rapidly formed but can dissociate very slowly after cleavage of the inhibitor at the peptide bond P1-P'1 of the active site located in the loop. Secretory leukocyte protease inhibitor and elafin are the two main extracellular canonical inhibitors identified in lung secretions (Hochstrasser, Reichert, Schwarz, & Werle, 1972; Sallenave & Ryle, 1991).

4.2 Maturation of mast cell serine proteases

The principal proteins of mast cell secretory granules are serine proteases, which are released outside of the cell by allergen-bound IgE as well as by cationic peptides and other stimuli (Douaiher, et al., 2014; Hellman & Thorpe, 2014). Most of these proteases are largely exclusive to mast cells, and several have been targeted for therapeutic inhibition (Caughey, 2016). The presence and abundance of proteases are signature features of mast cells, which contribute to IgE-driven chronic allergic diseases, including asthma, rhinitis, conjunctivitis and esophagitis, as well as to urticaria, anaphylaxis, non-allergic anaphylactoid reactions, mastocytosis, and mast cell activation disorders (Siebenhaar, Redegeld, Bischoff, Gibbs, & Maurer, 2017; Theoharides, Valent, & Akin, 2015). Mast cells also contribute in less well understood ways to mammalian host defenses, e.g., against venoms and intestinal parasites in mice (Metz, et al., 2007). Several mast cell proteases are activated from inactive zymogens in whole or part by CatC. Therefore, CatC blockade has the potential to alter mast cell function. Evidence supporting CatC-mediated activation of specific mast cell proteases is discussed below.

As a group, the proteases are thought to act mostly outside of the cell after secretion and release into the neutral to alkaline environment of extracellular fluids. The classic mast cell exocytotic pathway liberates granule contents from membranes during secretion. However, a fraction of proteases are released in membrane-bound microvesicles, which may be another way mast cells communicate with other cells (Groot Kormelink, et al., 2016). Although granule-associated mast cell serine proteases mostly lack established intracellular roles, they are stored pre-processed from zymogen precursors. However, activity in the membrane-bound interior is limited by low pH and tight packing in sequestered matrices of heparin and related polyanions, which reduce solubility, mobility and access to potential target peptides and proteins (Goldstein, Leong, Schwartz, & Cooke, 1992; Humphries, et al., 1999; Sayama, Iozzo, Lazarus, & Schechter, 1987). In support of its suspected importance in producing active secretory proteases, CatC is abundantly and differentially (albeit non-exclusively) expressed in mast cells and mast cell-derived tumors. Indeed, expression is sufficiently abundant in canine mastocytomas that it was purified and its sequence determined from cDNA cloned from that source (Wolters, et al., 1998). Tissue surveys provide evidence of differential expression of

CatC in mast cells. For example, mast cells are dominant CatC-expressing cells in the uninflamed dog airway (Wolters, Laig-Webster, & Caughey, 2000) and in mouse skin (Ruffell, et al., 2013). The subcellular sites where CatC processes specific mast cell zymogens remain to be established. Studies with cultured cells suggest that a portion of CatC is packaged in serine protease-rich mature granules (or in functionally similar compartments) and is secretable (Wolters, et al., 1998). The ability to store CatC in secretory granules may be one reason that mast cells are prominent sources of the enzyme in histochemical surveys. However, whether CatC released into extracellular environments rich in cystatins and other potential inhibitors will have the opportunity to act outside of the cell by cleaving extracellular targets remains unclear.

Mast cell chymases: The term "chymase" classically refers to serine proteases of mast cells with chymotrypsin-like ability to cleave peptide and protein targets after aromatic amino acids. Actually, mast cell chymases are less closely related to digestive enzymes like pancreatic chymotrypsin than to immune cell proteases, such as CatG, NE, and lymphocyte granzymes (Ahmad, Bird, & Kaiserman, 2014; Hellman & Thorpe, 2014; Zamolodchikova, et al., 2005). Moreover, substrate preferences of chymases differ from those of chymotrypsin, and some "chymases", having been affected by specificity-altering mutations in the vicinity of the substrate binding pocket, have little or no chymotryptic activity, but possess elastase, Leu-ase, or Met-ase activity, or have become catalytically inactive (Kunori, et al., 2002) (Caughey, et al., 2008). Along with granzymes, mast cell chymases are among the most varied groups of mammalian proteases with respect to number of expressed genes in a given species as well as their range of target specificities of chymases within and between mammalian species (Puente, et al., 2003).

In humans, fortunately for those trying to assign functions and identify drug targets, there is just one chymase gene (CMA1), which encodes a chymotryptic chymase. Human chymase is expressed and stored in granules only in a subset of mast cells, and is especially abundant in skin (Irani, Bradford, Kepley, Schechter, & Schwartz, 1989). Many mast cells residing near mucosal surfaces express little or no chymase. Although human chymase is capable of hydrolyzing many peptides and proteins typically after Phe or Tyr, and less often Trp or Leu-angiotensin II-generating activity is a

standout feature (Caughey, Raymond, & Wolters, 2000; Sanker, et al., 1997). While sharing the ability to convert angiotensin I to active angiotensin II, chymase is unrelated to angiotensin converting enzyme, which is a metallo-exopeptidase, not a serine protease. After release, active human chymase is captured in extracellular fluids by α2-macroglobulin, and loses the ability to cleave protein-size targets (Raymond, et al., 2009). However, while bound to α2-macroglobulin and circulating in blood, human chymase remains enzymatically active and retains its ability to generate angiotensin II. One goal of pharmaceutical efforts to develop chymase inhibitors is to prevent chymase-mediated generation of angiotensin II, with its potential role in promoting hypertension, cardiovascular remodeling, and organ fibrosis. Human chymase, like several other immune peptidases, is thought to be activated intracellularly by CatC. Human pro-chymase zymogen has an amino-terminal, acidic dipeptide activation sequence that remains after removal of its signal peptide. CatC can remove this activation sequence *in vitro* from pro-chymase (McEuen, Ashworth, & Walls, 1998). Although the segment is very short relative to many serine protease pro-peptides, the effect of removal on conformation and activity of human chymase is profound, as revealed by comparison of crystal-based structures and peptidase activity of pro-chymase versus activated, mature human chymase (Pereira, et al., 1999; Reiling, et al., 2003) (**Figure 9**).

Rats and mice differ from humans in expressing several chymase-like peptidases, some of which differ substantially from the lone human enzyme in substrate preferences and patterns of expression in specific microenvironments (Caughey, 2011). This multiplicity and variability are challenging for those attempting to use classic gene knockout strategies to establish functions for specific rodent chymases and to predict biological roles of human chymase (Bankova, et al., 2014; Pejler, Ronnberg, Waern, & Wernersson, 2010). Several lines of evidence suggest that the mouse Mcpt4 gene product, which is mast cell protease 4 (MCP-4), is most similar to human chymase in chymotryptic activity, including angiotensin II-generating capacity (Lundequist, Tchougounova, Abrink, & Pejler, 2004) and patterns of expression in mast cell subsets; therefore, many genetic deletion studies focus on MCP-4 (Gendrin, et al., 2017). Mice also express major chymotryptic protease MCP-1 in mucosal mast cells (which rarely express chymase in humans). MCP-1 appears to be important in expelling certain intestinal parasites (Knight, Wright, Lawrence, Paterson, & Miller,

2000). Curiously, the mouse gene product MCP-5, which is genetically and structurally most similar to the human enzyme, is elastolytic rather than chymotryptic (Kunori, et al., 2002), and thus receives less emphasis as a potential proxy for the human enzyme. Studies in mice and cultured murine mast cells lacking CatC suggest that chymotryptic as well as elastolytic murine chymases depend on CatC for processing and activation (Wolters, Pham, Muilenburg, Ley, & Caughey, 2001). Neither dermal mast cells, which express MCP-4, nor stomach mast cells, which express MCP-1, contain chymotryptic activity in CatC-deficient mice, as assessed by esterase histochemistry (Wolters, et al., 2001). Despite the lack of chymotryptic activity, mast cells are normally abundant in CatC-deficient mice, are well-granulated, and contain immunoreactive chymase protein. Mast cells cultured from the bone marrow of CatC-deficient mice express normal levels of elastolytic chymase MCP-5, which however, runs more slowly on SDS-PAGE gels consistent with unprocessed pro-enzyme, with CatC-expressing cells appearing to contain MCP-5 exclusively in its mature form (Wolters, et al., 2001). The zymogens of these chymases feature acidic pro-dipeptides: Gly-Glu for MCP-1 and MCP-4, and Glu-Glu for MCP-5, as for human chymase (**Table 3**). Studies with synthetic peptidic substrates based on chymase and granzyme sequences (Tran, et al., 2002) suggest that dipeptides ending in Glu are good but not exclusive substrates for CatC.

Mast cell CatG: Although human CatG is well known as a product of neutrophils and a constituent of NETs, it is also expressed in the same subset of human mast cells expressing chymase, at similar levels, and is packaged in the same secretory granules (Schechter, et al., 1990). CatG is the human protease most closely related to chymase, and its gene (CTSG) and the chymase CMA1 gene are next-door neighbors (Caughey, et al., 1993). Like chymase, CatG is highly cationic on its surface, and will bind strongly to polyanionic matrix of mast cell granules (Hof, et al., 1996; Kalupov, et al., 2009). CatG is also a product of rodent mast cells, although rather little is known of its subset expression and mast cell-specific roles. A single gene encodes CatG in murine and human genomes, respectively. However, as with chymases, potentially important functional differences separate rodent and human enzymes. Murine CatG is highly active with narrow target specificity typical of a chymotryptic serine protease (Kalupov, et al., 2009; Raymond, et al., 2010). Functionally, it is more

similar to human chymase than to human CatG, which is a sluggish enzyme of broader specificity, including chymotryptic, tryptic, "Met-ase" and "Leu-ase" activities (Polanowska, et al., 1998; T. Tanaka, Minematsu, Reilly, Travis, & Powers, 1985). The weakening of activity and potentially dangerous loss of specificity appear to be due to an active-site mutation introduced during evolution of higher primates and is not present in most mammals, including mice (Raymond, et al., 2010). Little is known about activation of CatG in human mast cells specifically. However, studies in rodent mast cell lines suggest that CatC is important and that activated CatG is directed to secretory granules (Dikov, Springman, Yeola, & Serafin, 1994; Gullberg, Lindmark, Nilsson, Persson, & Olsson, 1994). Given that human CatG's Gly-Glu pro-dipeptide is identical to that of chymase (**Table 3**), and that the proteases end up in the same granules, it is reasonable to hypothesize that they share biogenetic mechanisms, including dependence on intracellular processing and activation by CatC.

Mast cell tryptases: Tryptases are serine proteases expressed by mast cells and to a lesser extent by basophils. They are products of multiple genes and occur in a variety of molecular guises, including classical soluble, heparin-bound, inhibitor-resistant oligomers, but also as type I transmembrane proteins with a C-terminal anchor, and as circulating monomeric pro-enzymes. Although they are named based on trypsin-like preference for cleaving target peptides after basic amino acids, they are much more closely related to transmembrane prostasin, marapsin and testisin than to pancreatic trypsin and are more discriminating and less destructive than trypsin (Caughey, Raymond, Blount, et al., 2000; Trivedi, Tong, Raman, Bhagwandin, & Caughey, 2007). All or almost all mast cells in human tissues express tryptases, which are usually the most abundant proteins in the cells, where they are packaged in pools of exocytotic granules. Like chymases, tryptases are synthesized initially as zymogens but appear in granules in mature, pre-activated forms. However, CatC's role and importance in activation is less definitive for tryptases, which have longer pro-peptides than do CatC-activated chymase, CatG, NE, and granzymes.

In humans, the major active soluble tryptases are β-tryptases, which are products of adjacent genes TPSAB1 and TPSB2. Early studies identified a potential pathway for removal of the 8- to 10-residue pro-peptide involving tryptic auto-hydrolysis at Arg-3, followed by removal of the residual

Val-Gly dipeptide by CatC, or processive removal of the propeptide in dipeptide units. More recent studies suggest that CatC's role may be redundant, i.e., perhaps sufficient but not necessary for full maturation of β-tryptases in human mast cells, with one or more other thiol cathepsins being capable of completing the task (Le, Gomez, et al., 2011; Le, Min, et al., 2011). It remains possible that CatC inhibitors influence levels of active β-tryptases in humans, which exhibit substantial individual variation in number of active β-tryptase genes. Many humans, for example, inherit one or more alleles at the TPSAB1 locus encoding largely inactive α-tryptase, rather than an active β (Trivedi, Tamraz, Chu, Kwok, & Caughey, 2009). Also, inheritance of an inactive βIII-tryptase allele is common in some populations. Individuals inheriting a small number of active tryptase genes could be more susceptible to CatC blockade than individuals inheriting a full complement of four active β-tryptase genes.

Experiments in animal models further support a role for CatC in regulating levels of soluble tryptases. In mice lacking CatC, tryptase activity is reduced but not absent (Wolters, et al., 2001). In mast cells differentiated in culture from CatC-null mice, tryptase activity is reduced 75%, which appears to be due to a decreased content of mature tryptase MCP-6. This is consistent with partial dependence on CatC. However, unlike for mouse chymases, which appear nearly completely dependent on CatC for activation, there is no evidence of incompletely processed MCP-6 tryptase pro-forms in mast cell lysates. Further evidence of a role for CatC comes from studies of septic peritonitis in CatC-deficient mice (Mallen-St Clair, Pham, Villalta, Caughey, & Wolters, 2004). This model suggests that CatC in mast cells renders sepsis more likely to be lethal, possibly by activating MCP-6, which in turn destroys IL-6, which is a survival factor upon which beneficial effects of CatC deficiency depend.

Basophils in mice express tryptase MCP-11 (Ugajin, et al., 2009), also known as mastin, which is also highly active and well-characterized in dogs (Raymond, Sommerhoff, & Caughey, 2005). In humans, however, mastin is a non-transcribed pseudogene, and human basophils can be regarded as relatively protease-deficient, expressing β-tryptases at <1% of levels achieved by mast cells (Jogie-Brahim, Min, Fukuoka, Xia, & Schwartz, 2004). CatC's involvement, if any, in mastin activation is

unknown, but is a potential confounder in modeling involvement of human CatC and tryptases in mice. The extent of participation by CatC in the activation of membrane-anchored tryptases, namely γ/transmembrane tryptase, which is the product of a gene present in the genome of many but not all mammals, including humans and mice (Trivedi, et al., 2007; Wong, et al., 1999), is likewise unknown. Substantial differences in activation sequences between soluble tryptases/mastins and membrane-anchored tryptases suggest that other pathways may be involved.

An uncertain but possibly substantial proportion of tryptases released by mast cells are monomeric, catalytically inactive zymogen, which is suspected to be secreted constitutively and to be the form of nearly all immunoreactive tryptase circulating under baseline conditions (Akin, et al., 2007; Schwartz, et al., 2003). Whether CatC secreted by mast cells or other cell types could process tryptase from this extracellular pool and whether CatC processed monomeric tryptase could form active tetramers *in vivo* in the absence of granule heparin is presently unclear.

Other peptidases: CatC can influence other mast cell peptidases. For example, levels of carboxypeptidase A3, which can act in tandem with chymases to inactivate toxic endogenous peptides and venoms (Lundequist, et al., 2004; Metz, et al., 2006), are increased in mast cells from CatC-deficient mice (Henningsson, Wolters, Chapman, Caughey, & Pejler, 2003). This effect could be direct, by processing precursors of active carboxypeptidase A3, or indirect, by influencing levels of other enzymes activating or degrading carboxypeptidase A3. Studies in cultured murine mast cell lines indicating that pro-carboxypeptidase A3 and pro-chymase are activated by separate mechanisms, and that CatC plays little if any role (Dikov, et al., 1994), suggest that an indirect effect is more likely. Mast cells of humans and mice can also express granzymes, the activation of which presumably would be affected by inhibition of CatC (McGuire, Lipsky, & Thiele, 1993; Ronnberg, et al., 2014; Strik, et al., 2007; Wroblewski, et al., 2017)

5. CATC TARGETS AND THEIR PATHOPHYSIOLOGY

5.1 NSPs in chronic inflammatory lung diseases

Chronic obstructive pulmonary disease (COPD), cystic fibrosis, AAT deficiency (AATD) and idiopathic pulmonary fibrosis are chronic inflammatory respiratory diseases that affect over 1 billion people worldwide, and cause the death of 4 million people every year. A further increase in the number of deaths from lung diseases is predicted between now and 2020, in particular from COPD. The total financial burden of lung diseases in Europe amounts to nearly €102 billion (www.european-lung-foundation.org) with COPD contributing to almost one-half of this figure, followed by asthma and pneumonia. Current therapies fail to control these diseases for the majority of patients, and there is a need for new therapies that go beyond symptomatic relief and which reduce disease progression. A major contributor to the paucity of efficacious drugs is a lack of sufficiently predictive preclinical models of respiratory disease. Drugs that perform well in these preclinical models often fail in human clinical studies.

Circulating neutrophils carry the largest pool of already activated NSPs and, therefore, have a high destructive potential in various neutrophil-driven inflammatory diseases, such as COPD, cystic fibrosis, bronchiectasis, idiopathic interstitial pneumonia, respiratory distress syndrome in preterm babies, periodontal disease or psoriasis. Extracellular activities of NSPs are usually controlled by various endogenous inhibitors, such as AAT, in plasma, interstitial fluids, in the inflammatory exudate and in glandular secretions (Korkmaz, et al., 2010; Korkmaz, Kellenberger, Viaud-Massuard, & Gauthier, 2013; Korkmaz, et al., 2016). These inhibitors are often inactivated or downregulated at sites of acute exacerbations or chronic inflammation.

Great interest in elastase-related NSPs was aroused by the early observations of Gross and colleagues in 1965 showing that intratracheal instillation of elastolytic activity of a plant-derived crude papain preparation caused anatomic changes resembling human emphysema (Gross, Pfitzer, Tolker, Babyak, & Kaschak, 1965). This experimental *in vivo* model for human emphysema was subsequently reproduced in many laboratories using highly purified porcine pancreatic elastase, human NE and lately authentic mouse NE in mice (Dau, Sarker, Yildirim, Eickelberg, & Jenne, 2015).

The second key pillar for the involvement of NSPs in chronic inflammatory lung diseases are empirical clinical observations in individuals with an inborn genetic AATD, which is after albumin the second most abundant plasma protein and a very effective inhibitor of NE and elastase-related serine proteases. Strongly reduced plasma levels of AAT are caused by the same homozygous Z-mutation (Glu342Lys) in 95% of all individuals with severe AATD (Ferrarotti, et al., 2012; Zorzetto, et al., 2008). The latter mutation predisposes homozygous carriers to early-onset emphysema. As AAT is the most efficient inhibitor of NE, but is also relevant for the termination of PR3 activities after granule secretion, excessive activities of these two NSPs are regarded as major pathogenic factors in chronic inflammatory lung diseases. This widely accepted view is supported by genetic association studies showing that lower levels of AAT and dysfunctional alleles of the AAT gene *SERPINA1* increase the individual risk for COPD and the long term decline of airflow functions (FEV1). The inhibitory effects of plasma on NSPs is complemented by another serpin, α1-antichymotrypsin (serpinA3) which primarily targets the second member of NSPs, CatG (Travis & Salvesen, 1983). Missense mutations in this serpin were shown to be associated with chronic lung disease in rare cases already more than 25 years ago (Poller, et al., 1992) suggesting that CatG also contributes to chronic inflammatory lung pathologies in humans. Intracellular inhibitors of the neutrophil, in particular serpinB1, are an important counterweight for granule-associated NSPs, which leak into the cytosol in aging neutrophils, and protect them against premature apoptosis and secondary necrosis in tissues. The life span and steady state density of neutrophils in lung tissues therefore is also determined by this dynamic equilibrium between NSPs of primary granules and intracellular serpins (Benarafa & Simon, 2017). Whether neutrophils die by apoptosis, pyroptosis, necroptosis, netosis or secondary necrosis (Jorgensen, Rayamajhi, & Miao, 2017) further determines the dynamics of their removal by macrophages and their destructive impact on lung tissues. PR3 accelerates the intrinsic spontaneous cell death pathways in aging neutrophils by cleaving procaspase 3 (Loison, et al., 2014), but other forms of spontaneous aging-related death occur as recently noticed (Teng, Luo, & Kambara, 2017). The extent of locally dying neutrophils in chronically inflamed lung tissue and secondary neutrophil necrosis may thus have a strong impact on irreversible lung tissue damage.

The old concept of a disturbed NE-AAT balance in lung emphysema has been expanded and encompasses the interplay of additional serine proteases and their inhibitors, like CatG and the cytoplasmic serpinB1 (Benarafa & Simon, 2017). Moreover, the proinflammatory effects of metalloproteinases have been shown to act upstream of NSPs and contribute to tissue damage by NE indirectly by inactivating serpins (Fortelny, et al., 2014; Liu, et al., 2000). Matrix metalloprotease 9 (MMP9) of neutrophils cleaves the reactive center loop of AAT and unleashes excessive NE activity. Similarly MMP8, known as neutrophil collagenase, inactivates AAT and enhances the activity of NE and other serine protease targets (Fortelny, et al., 2014). Proteases of different classes cooperate as an interconnected dynamic system and acquire proteolytic momentum by mutually destroying local inhibitors of other proteases. Besides MMP8 and MMP9 (Vissers, George, Bathurst, Brennan, & Winterbourn, 1988), MMP1 (Desrochers, Mookhtiar, Van Wart, Hasty, & Weiss, 1992; Desrochers & Weiss, 1988) and MMP12 of activate macrophages (Banda, Clark, & Werb, 1985) can inactivate AAT, but also CatL, a lysosomal cysteine protease, inactivates AAT (Johnson, Barrett, & Mason, 1986) and helps NSPs escape from the highly abundant and effective AAT. Self-cleavage of NE moreover transforms this protease into a two-chain isoform which is still active, but relatively resistant to inactivation by AAT (Dau, et al., 2015). This auto-processing step occurs near its active site after neutrophil activation and reduces the efficiency not only of natural, but also of synthetic NE inhibitors.

Deviations from the optimal balance between NSPs and AAT can be caused by environmental factors like smoking and air pollution, and inborn mutations in the AAT gene *SERPINA1* mentioned above, as well as by mutations of other genes directly or indirectly affecting the functional levels of AAT and NSPs. The effects of such mutations are weak and pleiotropic which sometimes can synergize, but also can neutralize each other. In a genome-wide association study (GWAS), the minor allele of the rs7151526 SNP (frequency of 0.066 in CEU) in the noncoding region of AAT was associated with PR3-ANCA positive granulomatosis with polyangiitis (GPA). This allele directly tested in the study is in strict linkage disequilibrium with the Z-allele (0.03 in CEUR) of the rs289929474 marker. Hence, heterozygous and homozygous carriers of the Z-allele may solely account for this genetic association with the *SERPINA1* locus. Likewise an association with a SNP in the *PRTN3* locus was found to be highly significant. Hence lower levels of AAT in Z- and ZZ-carriers

and increased or altered expression of the PR3 autoantigen under normal and inflammatory conditions appear to be a risk for this relapsing remitting autoimmune disease.

The net excess of proteolytic activities in the peri- and extracellular tissue space intensifies immune defense responses, but also tissue damage in the early stage, while proteolytic activities during the resolution of inflammation dampen and down-regulate pro-inflammatory signaling cascades. Although small molecule or peptide-based inhibitors against individual NSPs have been extensively explored in the past 50 years, efforts of the pharmaceutical industry so far created very little impact in the clinic except for AAT augmentation therapy in emphysema patients with congenital AATD (Polverino, Rosales-Mayor, Dale, Dembowsky, & Torres, 2017). Targeting all four NSPs simultaneously may generate greater beneficial effects in various pathological settings, and hence lowering the constitutively produced serine protease pool of neutrophils by CatC inhibitors holds great promise for the future.

5.2 NSPs in ANCA-associated vasculitis (AAV)

The vasculitidis comprise a heterogeneous group of systemic inflammatory blood vessel diseases. Because of different etiologies and pathogenesis, the current nomenclature for non-infectious vasculitidis is based on the predominantly affected vessel type (Jennette, et al., 2013). The spectrum ranges from large-vessel vasculitis involving large arteries such as aorta and its major branches to small-vessel vasculitis that affects small intraparenchymal arteries, arterioles, capillaries, and venules. The latter group includes diseases that are characterized by anti-neutrophil cytoplasmic antibodies (ANCA) that circulate in the blood of patients. AAV include granulomatosis with polyangiitis, microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, and a renal-limited variant featuring necrotizing crescentic glomerulonephritis (NCGN). PR3 and MPO are the major target antigens recognized by ANCA (Falk & Jennette, 1988; Goldschmeding, et al., 1989; Jenne, et al., 1990). GPA is mainly associated with the PR3-ANCA and MPA with MPO-ANCA. The incidence of AAV is approximately 20/million population (Scott & Watts, 2013) but was reported to be 33/million in some regions (A. Berti, Cornec, Crowson, Specks, & Matteson, 2017).

AAV are systemic diseases that frequently involve the kidneys, lungs, ear-nose-and-throat (ENT), skin, and eyes. However, autopsy studies and case reports indicate that these diseases affect basically every organ of the body. Although the AAV entities show a large overlap in clinical manifestations, a few distinctions were recognized (Hilhorst, van Paassen, Tervaert, & Limburg Renal, 2015). When compared to MPA, GPA more often affects the respiratory tract, the orbital cavity, and shows more pulmonary nodules, as opposed to rather diffuse infiltrates and fibrotic lung lesions that are more common in MPA. Moreover, MPO-AAV is more often limited to the kidneys compared to PR3-AAV (Falk, Hogan, Carey, & Jennette, 1990). The kidney is the organ that is most frequently involved independent of the AAV type. NCGN with rapidly progressive renal failure is the hallmark of renal vasculitis and is often the manifestation that triggers the correct diagnosis. Finally, patients with PR3-ANCA disease more often experience relapses compared to MPO-ANCA disease (Lionaki, et al., 2012; Walsh, et al., 2012).

AAV were invariably lethal diseases before the introduction of steroids and cytotoxic drugs. Current treatment strategies include a more aggressive induction therapy to induce remission. Induction protocols consist of steroids in combination with either cyclophosphamide or rituximab that targets CD20-expressing B cells. Most clinicians would add plasma exchange when patients show NCGN with rapidly-progressive renal failure or when pulmonary hemorrhage occurs. Once remission is achieved, immunosuppression is de-escalated and a maintenance therapy is initiated that employs either azathioprine or rituximab for at least 18 months. These measures reduce inflammatory mediators, remove ANCA, and delete precursors of ANCA-producing or ANCA antigen-presenting cells. All of these standard treatments effectively induce remission in approximately 70% of patients with active AAV but harbor their own adverse effects that contribute to mortality and morbidity (Flossmann, et al., 2011; Hruskova, et al., 2015; McGregor, et al., 2015). Moreover, none of these treatments targets ANCA-specific disease mechanisms.

5.2.1 NSPs in the pathogenesis of AAV

Biopsies from AAV patients show highly inflammatory small-vessel vasculitis with fibrinoid vessel wall necrosis and granulomata that are occasionally ill-defined. The lack of immune deposits in

these lesions lead to the term "pauci-immune" and suggested disease mechanisms distinct from immune-complex or anti-glomerular basement membrane antibody-mediated vasculitis. Important steps towards elucidation of these disease mechanisms were made with the initial characterization of circulating autoantibodies, namely ANCA, in the blood of patients with GPA and the subsequent identification of PR3 and MPO as the target autoantigens that are exclusively expressed by neutrophils and monocytes (Falk & Jennette, 1988; Goldschmeding, et al., 1989; Jenne, et al., 1990; van der Woude, et al., 1985). These findings placed both leukocyte types in the center of many researchers' interest. Investigations then demonstrated that ANCA was not only a disease marker but also triggered neutrophil signaling and activation. Moreover, ANCA-activated neutrophils damaged endothelial cells *in vitro* (Kettritz, 2012). In 2002, a murine disease model was successfully established providing firm evidence that ANCA, at least MPO-ANCA, are indeed pathogenic (Xiao, et al., 2002). Unfortunately, despite several attempts, no suitable PR3-ANCA model exists. ANCA interaction with PR3 is more complex than with MPO as the neutrophil-specific CD177 receptor is involved in PR3 surface expression and PR3-ANCA induced neutrophil activation (**Figure 10**). We generated double-transgenic mice that expressed human PR3 and CD177 under a myeloid-specific huMRP8 promoter in an attempt to model PR3-AAV. We found to our disappointment that anti-PR3 antibodies did not activate double-transgenic neutrophils (Schreiber, Eulenberg-Gustavus, Bergmann, Jerke, & Kettritz, 2016).

NSPs are active enzymes that are released from neutrophils and monocytes into the surroundings during cell activation. A variety of NSP-mediated effects have been characterized in general inflammatory conditions (Kettritz, 2016; Korkmaz, et al., 2010). However, NSPs were also studied in the ANCA context. Some of the reported NSP effects have implications that concern both PR3-ANCA or MPO-ANCA vasculitis, whereas PR3 has additional implications as an autoantigen that concerns specifically PR3-ANCA disease.

5.2.2 PR3 is the NSP that functions as a major autoantigen in AAV

PR3 provides a major autoantigen in AAV patients. PR3 is stored in intracellular granules and vesicles and is presented on the cell membrane of neutrophils. mPR3 is accessible to circulating PR3-

ANCA and PR3-ANCA binding to their membrane-expressed target antigen initiates activation of cytokine-primed neutrophils. Thus, understanding mechanisms that control mPR3 presentation is important.

PR3 is produced by all neutrophils. However, only a subset of these cells will express high PR3 levels on the outer cell membrane. As a consequence, a bimodal mPR3 pattern is found in more than 95% of the healthy people with the distinction of a $mPR3^{low}$ and $mPR3^{high}$ neutrophil subset (Halbwachs-Mecarelli, et al., 1995). The percentage of the latter ranges from a few to 100%, is stable in a given individual and does not change with neutrophil activation. PR3 presentation on the neutrophil surface occurs by at least two mechanisms, namely direct membrane insertion (Kantari, et al., 2011) and CD177 association (Bauer, et al., 2007; von Vietinghoff, et al., 2007). We characterized CD177, a neutrophil-specific surface receptor, to support the presentation of large PR3 amounts on the cell surface (von Vietinghoff, et al., 2007) and to inhibit PR3 activity (Jerke, Marino, Daumke, & Kettritz, 2017). CD177 shows a random monoallelic expression pattern that is controlled by DNA methylation and histone modifications (Eulenberg-Gustavus, Bahring, Maass, Luft, & Kettritz, 2017). Monoallelic CD177 expression is restricted to a variable, but stable neutrophil subset yielding distinct $CD177^{neg}/mPR3^{low}$ and $CD177^{pos}/mPR3^{high}$ neutrophils. Studies in AAV patients indicated that a higher percentage of $CD177^{pos}/mPR3^{high}$ neutrophils carries a risk for AAV and poorer clinical outcomes (Rarok, Stegeman, Limburg, & Kallenberg, 2002; Schreiber, et al., 2005; Witko-Sarsat, et al., 1999). Although PR3-ANCA can enter the cell, these clinical observations suggest that mPR3 provides an important step in initiating the PR3-ANCA:neutrophil interaction. In fact, PR3-ANCA induced a stronger respiratory burst in $mPR3^{high}$ compared to $mPR3^{low}$ neutrophils (Schreiber, Luft, & Kettritz, 2004). Because PR3 is not a transmembrane protein, additional adapter molecules are needed, including the β2 integrin CD11b/CD18 (Jerke, et al., 2011). In contrast to neutrophils, only small PR3 amounts are found on the surface of monocytes and the PR3-ANCA interaction with this cell type is less well characterized.

Surface-expressed PR3 is important for initiating PR3-ANCA-induced activation of viable neutrophils. In addition, large amounts of PR3 are found on the cell membranes of apoptotic neutrophils. Active PR3 is externalized to the outer cell membrane during apoptosis by a scramblase-

1-dependent mechanism. This mPR3 pool on apoptotic cells acts as a "do-not-eat-me" signal (Kantari, et al., 2007). As a consequence, apoptotic cell clearance by macrophages is reduced and a pro-inflammatory condition develops that promotes autoimmunity. Apoptotic rat basophilic leukemia cells that expressed enzymatically active PR3 on their cell surface caused macrophages to generate high amounts of inflammatory cytokines and chemokines (Millet, et al., 2015). Moreover, pDCs that were exposed to this milieu promoted CD4 T cell polarization. Importantly, the presence of PR3-ANCA polarized this process towards a Th17-cell distribution. This finding could be relevant for AAV that is manifest with crescentic glomerulonephritis, because Th17 cells were shown to contribute to crescent formation, at least in non-ANCA murine disease models (Disteldorf, et al., 2015; Odobasic, et al., 2011; Tulone, Giorgini, Freeley, Coughlan, & Robson, 2011). The importance of Th17 cells in an ANCA-induced NCGN model has not yet been established. However, increased IL-17A levels were found in sera from GPA patients. The findings suggest that Th17 cells play a disease-relevant role (Millet, et al., 2015; Wilde, et al., 2012).

5.2.3 NSPs function as effector molecules mediating injury in vasculitis

NSPs are active enzymes that not only proteolytically process a variety of substrates but also exert biological effects not requiring enzymatic activity (Kettritz, 2016; Korkmaz, et al., 2016). We will focus on NSP effects that were specifically characterized in an ANCA context.

NSPs contribute to endothelial damage: During neutrophil activation, NSPs are translocated from intracellular granules and vesicles to the cell membrane and are released into the surroundings, either as soluble molecules or tethered to NETs. We showed previously that ANCA-stimulated neutrophils degranulated NSPs that were then internalized by neighboring endothelial cells (Jerke, et al., 2015). Importantly, the transfer from the neutrophil to the endothelial cells did not compromise the proteolytic activity of the NSPs. We characterized the cleavage pattern for each NSP, generated IceLogos, and identified common and specific endothelial substrates using terminal-amine isotopic labeling of substrates. Gene ontology analysis showed an enrichment for cytoskeletal proteins. We showed subsequently that incubation of an endothelial monolayer with supernatants from ANCA-

activated neutrophils caused an increase in albumin flux and disturbed the endothelial architecture. Both effects were prevented by pharmacologic serine protease inhibition. These data suggest that NSPs contribute to the ANCA-mediated endothelial inflammation and damage.

NETs contain, in addition to DNA, histones, and granular proteins. NSPs, as was shown for NE, participate in NET formation by promoting chromatin decondensation (Papayannopoulos, et al., 2010). Moreover, NSPs in the extracellular space associate with NET structures. In a proteomic analysis, NE was the most abundant non-histone protein in NETs, whereas CatG and PR3 amounts reached approximately 40% and 10%, respectively (Urban, et al., 2009). Profiling NET-associated enzymatic activity showed that NE provided the vast majority of proteolytic activity (O'Donoghue, et al., 2013). Initial observations in renal biopsies from patients with active AAV visualized NETs in crescentic glomeruli and showed that ANCA caused NET formation *in vitro* (Kessenbrock, et al., 2009). In addition, these glomerular NETs contained LL37, a granule protein that is implicated in the conversion of self-DNA into a pDC activator. The combination of DNA, PR3 and LL37 would possibly promote the "break-of-tolerance" towards PR3. A similar mechanism may occur in MPO autoimmunity because MPO is found in NETs as well.

We showed recently that ANCA induce NETs by receptor-interacting protein kinase (RIPK1/3)- and mixed lineage kinase domain-like (MLKL)-dependent necroptosis (Schreiber, et al., 2017). Moreover, these NETs provided a scaffold for the activation of the alternative complement pathway that led to C5a generation. These experiments revealed a novel mechanistic link between ANCA-induced NET formation and alternative-complement pathway activation. Moreover, NETs induced endothelial cells damage *in vitro* in a complement-dependent manner. These findings are clinically relevant because we and others demonstrated that C5a interaction with the activating C5a receptor (CD88) accelerated ANCA-induced neutrophil activation *in vitro* and caused NCGN in murine disease models (Schreiber, et al., 2009; Xiao, et al., 2014). Very recently, C5a receptor blockade by the small inhibitory compound avacopan was shown to effectively induce remission patients with active AAV (Jayne, et al., 2017; Kettritz, 2017). Together, these data show that active NSPs contribute to inflammation and injury in several ways, including proteolytic degradation of cellular structure

proteins, NET formation, NET-mediated complement activation, and possibly NET-mediated contributions to the loss of tolerance.

NSPs control the production of pro-inflammatory mediators: NSPs shape the inflammatory response by processing extracellular matrix proteins, cytokines and chemokine, growth factors, and receptors (Kettritz, 2016). We assume that at least some of the effects that are at work in general inflammation are relevant to the highly inflammatory AAV setting. One example where this assumption was confirmed is provided by IL-1β. This molecule is strongly increased in a variety of infectious and non-infectious inflammatory conditions, including sepsis, gout, cryopyrin-associated periodic syndromes. IL-1β is also increased in ANCA vasculitis (Noronha, Kruger, Andrassy, Ritz, & Waldherr, 1993). IL-1β production is controlled by IL-1β converting enzymes that process the inactive 31 kDa precursor into the 17 kDa mature fragment (Thornberry, et al., 1992). Caspase-1 was characterized as the classical IL-1β converting enzyme but additional converting enzymes were suspected, including NSPs. When we stimulated human and murine monocytes and neutrophils with ANCA, we observed strong IL1-β generation that was significantly higher in monocytes compared to neutrophils (Schreiber, et al., 2012). We used cells from wilde-type and gene-deficient mice and observed that IL-1β production was strongly reduced with either CatC or combined NE/PR3-deficiency. This reduction was reversed when active PR3 was added to the cells before activation. IL-1β was also reduced when ANCA-stimulated normal human neutrophils were incubated with a cell-permeable serine protease inhibitor. These data establish an important role for NSPs in ANCA-induced IL-1β generation.

NSPs control ANCA-induced vasculitis in a murine disease model: CatC-deficient mice lack proteolytic activity of all three NSPs whereas reduction of protein levels varied depending on the NSP type (Adkison, et al., 2002). We employed CatC-deficient mice to study NSPs in the *in vivo* complexity of a murine ANCA disease model (Schreiber, et al., 2012). In this model, MPO-deficient mice were immunized with murine MPO to induce anti-MPO antibodies. After irradiation, bone marrow from either wild-type of CatC-deficient mice were transplanted. After marrow transplant, all

mice that received wild-type bone marrow developed renal vasculitis with severe NCGN. In contrast, mice that received bone marrow from CatC-deficient animals were protected from disease. The protective effect was, at least in part, mediated by IL-1β reduction. This conclusion was derived from the fact that an IL-1 receptor antagonist protected mice that had received wild-type bone marrow and that CatC deficiency reduced ANCA-induced IL-1β generation in monocytes and neutrophils together with renal IL-1β levels. It should be noted that CatC-deficient mice show also reduced tryptase (Wolters, et al., 2001) and granzyme (Pham & Ley, 1999) activity indicating that CatC controls additional proteases. However, we reproduced key findings in our disease model that were obtained with CatC deficiency with combined NE/PR3-deficiency suggesting that NSPs contributed significantly to the disease, at least in part, by IL-1β dependent mechanisms.

NSP aberrations in humans with implications for ANCA vasculitis: Patients with PLS harbor mutations at the CatC gene locus (Hart, et al., 1999; Toomes, et al., 1999). The resulting lack of active CatC leads to strong reduction in enzymatic NSP activity and protein stability (Pham, et al., 2004). A recent analysis documented the complete absence of CatC and mature NSPs from neutrophil granule preparations obtained from a PLS patient (Sorensen, et al., 2014). The investigators tested several neutrophil responses and observed that NET generation was strongly reduced. Additional interesting observations resulted from this carefully performed patient study. PLS neutrophils showed a major reduction in the generation of LL37 from hCAP-18. As mentioned above, LL37 could promote autoimmunity towards PR3, and was found together with PR3 in NETs (Kessenbrock, et al., 2009) and in granulomatous lesions of GPA patients (Millet, et al., 2015). Since MPO also associates with LL37-decorated NETs, the same could apply to anti-MPO autoimmunity. The PLS patient-derived findings suggest that NSPs control the formation of NETs that have several implications for AAV, including endothelial damage, complement activation, and the breaking of immune tolerance.

Are there any data that indicate NSP aberrations in AAV patients? Two large genome-wide association studies (GWAS) established a highly significant association between PR3-ANCA vasculitis and single nucleotide polymorphisms in the genes for PR3 and AAT (P. A. Lyons, et al., 2012; Merkel, et al., 2017). Although association studies cannot establish causal relationships, the data

suggest a mechanistic role for PR3 as an active NSP in the disease. The biological meaning of these polymorphisms needs to be determined. Another NSP-related finding was derived from AAV patient material. NSPs are transcribed at early stages of neutrophil differentiation whereas mature neutrophils have completely silenced this transcription process. However, when mature blood neutrophils were analyzed in patients with active AAV and controls, active NSP transcription was found in the former but not in the latter (Yang, et al., 2004). Subsequent studies showed that increased H3K27me3-specific demethylase jumonji C domain-containing protein 3 (JMJD3) expression and Runt-related transcription factor 3 (RUNX3) promoter methylation counteracted PR3 gene silencing (Ciavatta, et al., 2010). Moreover, the aberrant PR3 transcription generated alternative transcripts in some patients with active AAV, independent of the ANCA specificity (McInnis, et al., 2015). PR3-ANCA from some of these patients recognized a 24-kDa PR3 protein that was derived from an alternative transcript suggesting that reactivated PR3 transcription could lead to PR3 fragments with antigenic properties.

In summary, ANCA-activated neutrophils and monocytes mount a variety of responses that depend on NSPs. These responses contribute in many ways to the highly inflammatory milieu that is characteristic of AAV. In addition to inflammation, NSPs promote autoimmunity on several levels. Conceivably, these NSP-related mechanisms concern both PR3- and MPO-AAV. PR3 is unique amongst the NSP family members for it has an additional role by providing a major autoantigen in AAV. We speculate from the available data that mechanisms that reduce NSP proteins or activity could become a novel treatment option in AAV. Future studies should identify the best pharmacologic strategy to effectively target NSPs.

5.3 Mast cell proteases in asthma

Mast cells are strongly implicated in the pathophysiology of allergic diseases in general and asthma in particular. Multiple classes of therapeutic agents including mast cell-stabilizing cromones, anti-IgE, blockers of lipoxygenase and prostaglandin pathways, inhibitors of mast cell growth and signal transduction, and anti-histamines reduce mast cell numbers, activation or untoward effects of secreted products (Siebenhaar, et al., 2017). Being major secreted products of mast cells, and immediately active upon release, serine proteases have been considered as targets for development of

drugs to prevent or relieve asthma. As discussed below, present evidence more robustly supports roles for tryptases than for human CatG or chymase. As noted elsewhere in this review, CatG and chymases are more dependent on CatC for activation than are tryptases, and thus can be expected to be more thoroughly inactivated in mast cells by inhibitors of CatC. We might look to individuals naturally lacking CatC, such as those with PLS, for guidance in this regard. Presently, however, there is no compelling evidence that PLS is associated with increased or diminished asthma incidence or severity, but the number of these individuals is small and little is known of the medical histories of most of them, so this cannot be regarded as a strong predictor of effects of CatC inhibition on asthma. Indeed, with respect to mast cells, it remains to be shown that levels of active mast cell granule proteases are reduced in PLS.

5.3.1 Tryptases

Tryptases were the mast cell proteases first targeted for pharmaceutical intervention in allergic diseases and asthma (Cairns, 2005; Caughey, 2016)). This was because human mast cells in nearly every tissue location express tryptases in abundance and release them into nasal and bronchial passages in response to mast cell stimulation with aeroallergen (Wenzel, Fowler, & Schwartz, 1988). Just as significantly, some mammalian tryptases act in ways suggesting potential contributions to short-term bronchoconstriction and long-term airway remodeling (Ruoss, Hartmann, & Caughey, 1991; Sekizawa, Caughey, Lazarus, Gold, & Nadel, 1989; Tam & Caughey, 1990). However, tryptases pose challenges to drug development, among them the multiplicity of human alleles and genes (Trivedi, et al., 2007), variation in the number of inherited functional genes (Abdelmotelb, et al., 2013; J. J. Lyons, et al., 2016; Sabato, et al., 2014; Trivedi, et al., 2009), and storage of tryptases in exceptionally high amounts within membrane-bound organelles, allowing the concentration of tryptases in the vicinity of degranulating mast cells to reach very high local levels relative to clinically achievable concentrations of inhibitor drugs. Because tryptases are stored pre-activated in granules at low pH, there is the challenge of developing drugs that can penetrate cell and granule membranes and act in an acidic environment in which the local concentration of protease is very high, and yet continue to block tryptase activity when granule contents are released into neutral or alkaline environments

outside of cells. For active-site and mechanism-based inhibitors, there is the additional challenge of avoiding off-target effects affecting one or more of the large number of tryptic serine proteases in the mammalian proteome, including proteases associated with digestion, complement activation, clotting, fibrinolysis, ion flux and epithelial differentiation. Finally, phenotypes in mice genetically deficient in tryptases raise concern about potentially deleterious effects of systemic inhibition, including enhanced susceptibility to bacterial infection (Thakurdas, et al., 2007). Nonetheless, experiments involving animal models and small trials in humans suggested the possibility that inhibition of tryptase could prevent or relieve asthma or nasal allergy (Chen, et al., 2006; Clark, et al., 1995; Costanzo, et al., 2008; Krishna, et al., 2001). However, some of the compounds used in these studies were of suboptimal potency and specificity and fail to provide entirely convincing validation of tryptase inhibition as a strategy for treating asthma.

In some respects, especially regarding the potential of preventing active tryptases from accumulating in granules in the first place, CatC inhibition offers an attractive approach. However, based on data summarized earlier in this review, it is not clear to what extent human tryptase levels would be affected by CatC inhibition. Extrapolating from studies in mice lacking CatC and human mast cells in culture, the most likely outcome is partial reduction of active soluble (β) tryptase. There is also the potential of an effect on levels of active transmembrane/γ-tryptase, which provokes hyper-responsiveness when the recombinant soluble human enzyme is placed in a mouse airway (Wong, et al., 2002). However, it remains to be determined whether 1) CatC activates γ-tryptase and 2) the native, membrane-anchored enzyme causes hyper-responsiveness in murine or human airways. To add to the uncertainty, γ-tryptase (Prss31)-deficient mice have greater rather than less airway responsiveness to methacholine, although they are partially protected from the effects of cigarette smoke on lung and from dextran sodium sulfate-induced colitis (Hansbro, et al., 2014).

5.3.2 Chymase

As outlined in the preceding section, it is likely that a fully effective inhibitor of CatC will reduce and possibly nearly eliminate mature, active chymase in mast cells. Apart from any effects mediated by reduction of active mast cell tryptases, how blockade of CatC-mediated activation of pro-

chymase would influence airway obstruction in asthma is challenging to predict because a role, if any, of human mast cell chymase in asthma remains to be established. Studies in mice suggest that chymase can either promote or diminish asthma-like airway responsiveness. For example, the chymotryptic mucosal mast cell chymase MCP-1 appears to promote bronchoconstriction (Sugimoto, et al., 2012), whereas the connective tissue chymase MCP-4 appears to diminish it (Waern, et al., 2009). Mast cells migrating into human airway epithelium in mild to moderate asthma correlate with the corticosteroid-responsive "Th2" endotype and have the unusual phenotype of being tryptase- and mast cell carboxypeptidase A3-positive but chymase-low or -negative (Dougherty, et al., 2010). Similar phenomena occur in eosinophilic esophagitis-associated mastocytosis (Abonia, et al., 2010) and in mast cells infiltrating nasal polyp epithelium in chronic rhinosinusitis (Takabayashi, et al., 2012). In severe asthma, the number and percentage of chymase-containing mast cells in submucosal tissues and epithelium can increase (Fajt & Wenzel, 2013; Hinks, et al., 2015); whether chymase's presence in the asthmatic airway is helpful, harmful or indifferent is unclear. Because chymases studied *in vitro* can detoxify allergens (Mellon, Frank, & Fang, 2002) and inactivate allergic cytokines like IL-33 with a potential role in sustaining allergic inflammation (Roy, et al., 2014; Zhao, Oskeritzian, Pozez, & Schwartz, 2005), there are additional experimental reasons to consider beneficial roles for chymases in allergic diseases. Although potent, selective drug-like inhibitors of human chymase have been developed, the pharmaceutical emphasis to date has been on interfering with chymase-mediated generation of angiotensin II in hypertension (M. Li, et al., 2004), atherogenesis (Libby & Shi, 2007; Swedenborg, Mayranpaa, & Kovanen, 2011), and cardiovascular ischemic injury and remodeling (Oyamada, Bianchi, Takai, Chu, & Sellke, 2011; Wei, et al., 2010). Dual inhibitors of chymase and CatG have also been designed and synthesized. Consequences of CatC inhibition on mast cell serine protease activity potentially are even broader, with effects on one or more tryptases as well as on chymase and CatG.

6. SYNTHETIC INHIBITORS OF CATC TESTED IN PRECLINICAL AND CLINICAL STUDIES

Its strategic position during the maturation of NSP zymogens in hematopoietic bone marrow precursor cells makes CatC an attractive therapeutic target for neutrophil-dependent inflammatory diseases. Many research groups have focused their efforts on the design and development of potent and selective inhibitors of CatC (Guay, et al., 2009; D. I. Laine & Busch-Petersen, 2010). There are several classes of CatC inhibitors that have been described to date. Most of the reported inhibitors are based on the preferred dipeptide substrates and bear electrophilic warheads, forming either reversible or irreversible covalent bonds with the enzymes active site Cys234 (Guay, et al., 2009; D. I. Laine & Busch-Petersen, 2010).

Even though many potent and selective CatC inhibitors have been designed and developed, only a few reports have demonstrated biological effects *in vivo*. The major concern during the development of CatC inhibitors was their metabolic stability. The synthesized compounds were studied in different cell assays, rat liver microsomes and in rodents, and were often further optimized in order to increase their stability, which often came at the cost of the inhibitory activity. Very few compounds were advanced to *in vivo* studies, where their pharmacokinetic profile or biological effect was determined. Because the early activation of NSPs from their zymogens occurs already in bone marrow neutrophil precursors, effective CatC inhibitors need to reach their target in the bone marrow before CatC has activated the NSP zymogens. Nitrile inhibitors are reversible, consequently, measuring CatC activity in blood neutrophils is not sufficient to establish effective and biologically meaningful CatC inhibition. However, because effective CatC inhibition will subsequently result in NSP inactivation and zymogen degradation, both events reflect the intended biological consequences of CatC inhibition and may serve as biomarkers of effective pharmacological CatC inhibition (**Figure 11**).

Recently, a variety of CatC inhibitors was developed both by pharmaceutical companies and academic investigators, which are currently used and evaluated in preclinical and clinical studies.

6.1 Reversible nitrile CatC inhibitors

Nitrile compounds are the most studied group of inhibitors of cysteine cathepsins. The reaction between nitrile-based inhibitors and the active site Cys involves the reversible formation of a thioimidate adduct. Three chemical classes of nitrile-based inhibitors have been recognized so far: (i) cyanamides, (ii) aromatic nitriles and (iii) aminoacetonitrile derivatives which differ in their electrophilic properties. The nitrile group is not markedly electrophilic towards nucleophils, unless activated by adjacent structural elements such as electron withdrawing groups. In the case of simple arylnitriles, the electrophilicity is rather weak, thus the protease inhibition is also limited. Aminoacetonitriles exhibit an intermediate electrophilicity. Conversely some cyanamides and heterocyclic nitriles exhibit a strong electrophilicty, leading to a poor selectivity and non-specific tissue binding is often observed (Furber, et al., 2014).

AZD5248 and AZD7986: Most of the reported nitrile-based inhibitors of CatC are dipeptidyl nitriles. The first study was published in 2006 by Bondebjerg et al., after filing a patent in 2004 (Bondebjerg, Fuglsang, Valeur, Pedersen, & Naerum, 2006). The starting point of the study was a moderate CatC inhibitor, glycyl-L-phenylalanine nitrile (Gly-Phe-CN). The inhibitory activity towards CatC was improved by introduction of Abu in the P2 position, whereas various hydrophobic, aromatic amino acid residues such as Phe, biphenyl (Bip) were evaluated in P1 position. Abu-Bip-CN was identified as the most potent inhibitor in the series with an IC_{50} of 13 ± 3 nM (pIC_{50}= 8.7) (**Figure 12A**). The compound was found to be selective over CatB, H, and L. Despite its high inhibitory potency towards recombinant CatC, Abu-Bip-CN was poorly potent in the cell assay due to its rapid proteolytic withdrawal in the cell assay medium. The hydrolysis of the amide bond was also observed in plasma.

The study on Abu-Bip-CN and related compounds was continued by AstraZeneca and two strategies were employed to improve plasma stability (Furber, et al., 2014). Firstly, a piperidyl derivative of Abu-Bip-CN was obtained (compound **1**, (S)-N-((S)-1-cyano-2-(biphenyl-4-yl)ethyl)piperidine-2-carboxamide), (**Figure 12B** and **Figure 13A**). This compound was resistant to proteolytic degradation in plasma but was less potent than Abu-Bip-CN. Nevertheless, phenyl rings of

both compounds were oxidized in the presence of rat hepatocytes *in vitro*, confirming poor metabolic stability. In order to identify a suitable combination of both potency and stability, various substitutions of the biphenyl and piperidine rings were investigated. Interestingly, the incorporation of 4(S)-hydroxyl into the piperidine ring (compound **2** and **3**) resulted in an increased inhibitory potency (**Figure 12C,D**). The compound **3** ((2S,4S)-N-((S)-1cyano-2(4-(2-methyl-1-oxoisoindolin-5-yl)phenyl)ethyl)-1-hydroxypiperidine-2-carboxamide) exhibited an enhanced selectivity and was the most promising inhibitor in the series. The second strategy proposed to improve the metabolic stability, was the replacement of the N-terminal amino acid comprising the piperidine ring with an amino acid having a cyclohexyl ring as side chain. The yielded compounds were stable in both plasma and cell assays. The introduction of an oxygen atom within a new ring was the next modification, done to reduce both basicity and lipophilicity of the compounds. Compound **4** (AZD5248), bearing a simple nitrile substituent in the 4-position of the outer phenyl ring (S)-4-amino-N-(1-cyano-2-(4'-cyanobiphenyl-4-yl)ethyl)-tetrahydro-2H-pyran-4-carboxamide) was selected by Furber et al., as the most promising candidate for *in vivo* studies (Furber, et al., 2014) (**Figure 12E**). AZD5248 displayed a potent inhibitory activity and selectivity, and showed both a low clearance and a high bioavailability in rat, mouse and dog models. Gardiner et al., evaluated the downstream effect of CatC inhibition by AZD5248 (10 mg/kg) on NSPs activation *in vivo* in naïve rats (Gardiner, et al., 2016). AZD5248 or a vehicle control were administrated orally twice daily for 8 days. The maximal NSP inhibition was achieved in bone marrow after 8 days of treatment with 90%, 64% and 88% reduction in NE, PR3 and CatG activity, respectively. However, AZD5248 showed aortic binding in a rat quantitative whole-body autoradiography study, and its development was stopped prior to human dosing, despite the absence of obvious pathology in one month toxicology studies (Bragg, et al., 2015). It was hypothesized that aortic binding was mediated by imidazolin-4-one formation with aldehydes involved in the cross-linking of elastin, but no direct proof was presented, as the covalent AZD5248-elastin adducts were not identified.

A novel series of nitrile inhibitors free from aorta binding liabilities was developed. Doyle et al., identified compound **5** (AZD7986) (S)-N-((S)-1-Cyano-2-(4-(3-methyl-2-oxo-2,3-dihydrobenzo-[d]oxazol-5-yl)phenyl)ethyl)-1,4-oxazepane-2-carboxamide) as a highly potent, reversible and

selective inhibitor of CatC (Doyle, et al., 2016) (**Figure 12F**). This compound did not bind to aortic tissue homogenates and showed a good stability in plasma with a half-life of >10 h. AZD7986 inhibited almost completely activation of NE, PR3 and CatG in a concentration-dependent manner in human primary bone marrow-derived $CD34^+$ neutrophil progenitor cells (Doyle, et al., 2016). AZD7986 showed good species crossover for mouse, rat, dog and rabbit CatC. The downstream effects of CatC inhibition by AZD7986 on NSPs activation were assessed *in vivo* in naïve rats. Treatment of animals with AZD7986 twice daily for 8 days resulted in a dose-dependent decrease of NE and PR3 activity in bone marrow cell lysates. AZD7986 is the first nitrile inhibitor that reached clinical trials (Doyle, et al., 2016), and randomized, placebo controlled human phase 1 studies were started in 2014 (https://clinicaltrials.gov/ct2/show/NCT02303574). The assessment of the safety, tolerability and pharmacokinetics/pharmacodynamics of single and multiple oral doses of AZD7986 was performed in 81 healthy subjects (Palmer, et al., 2018). The impact of AZD7986 on whole blood NE activity was evaluated following 21 or 28 days of repeated dosing with 10, 25, or 40 mg AZD7986 or placebo. Daily dosing of 10, 25 and 40 mg AZD7986 resulted in 30, 49 and 59% reduction of NE activity (Palmer, et al., 2018). Several dose-dependent but non-serious skin manifestations, which are not correlated with the dynamics of NE activity, including both peeling and hyperkeratosis were observed. However, the symptoms were mild and not considered sufficiently important to prevent further clinical development (Palmer, et al., 2018). In 2016, the biotechnology company Insmed, Inc. announced a licensing agreement with the pharmaceutical company AstraZeneca for global exclusive rights to AZD7986. Insmed has renamed the compound INS1007 and will pursue an initial indication of non-cystic fibrosis bronchiectasis (http://investor.insmed.com/releasedetail.cfm?releaseid=992169).

Peptidyl cyclopropyl nitrile inhibitor: Another study investigating the structure-activity relationship of dipeptidyl nitriles as CatC inhibitors was conducted by researchers from the pharmaceutical company Merck & Co., Inc. (Guay, et al., 2009). A series of nitriles with β-(2-thienyl)-L-alanine at P2 position and various hydrophobic, aromatic residues were identified as very potent and selective CatC inhibitors (compound **6** and **7**) (**Figures 14A, B**). It was claimed that sulfur atoms were involved in a favorable binding interaction at the S2 site of CatC. Unfortunately, when the

obtained compounds were incubated with hepatocytes or administered intravenously to rats, they were rapidly degraded by hydrolases. It was demonstrated that increasing the steric bulk in P1 significantly improved hydrolytic stability, however, the potency against CatC was decreased. The modification that retained both potency and stability was the incorporation of a 1,1-cyclopropyl amino nitrile moiety at P1(compound **8** and **9**), with increased stability during incubation with hepatocytes (**Figures 14C, D**). Méthot et al., showed that compound **9** was able to inhibit almost completely the activation of NE, PR3 and CatG in a concentration dependent manner in cell-based assays using a promyeloid cell line (EcoM-G) (Methot, et al., 2007). These results were the first showing that near complete inhibition of NSPs can be achieved using a CatC inhibitor. In a human myelomonocytic cell line (U937), the processing of PR3 was prevented by the cysteine protease inhibitor E64d in culture, but not by a specific inhibitor of CatC (Rao, et al., 1996) or by compound **9** (Korkmaz et al., unpublished). However, the processing of NE and CatG was almost completely abolished by compound **9** (Methot, et al., 2007). In cellular assays using U937 and EcoM-G cells, NE protein levels were not lowered by CatC inhibition (Methot, et al., 2007).

The downstream effect of CatC inhibition by compound **9** on NSP activation was assessed *in vivo* in naïve rats. Animals were cannulated in the femoral vein and infused with compound **9** (5 mg/kg/days) or the vehicle alone. NSP activities were measured in blood leucocyte lysates after 2 weeks of infusion. Compound **9** did not significantly affect the percentage of white blood cells. NE, PR3 and CatG were significantly but not completely reduced by 80%, 70% and 50%, respectively.

IcatC (XPZ-01): A series of peptidyl cyclopropyl nitrile compounds as putative CatC inhibitors derived from compound **1** was designed and developed by the biotechnology company UNIZYME Laboratories. One of these compounds ((S)-2-amino-N-((1R,RS)-1-cyano-2-(4′-(4-methylpiperazin-1-ylsulfonyl)biphenyl-4-yl)cyclopro-pyl)butanamide)) (compound **10**) was identified as a cell-permeable, reversible, potent and selective inhibitor of human CatC with an IC_{50} value of ~15 nM in a CatC inhibition assay using glycyl-L-phenylalanyl-p-nitroanilide as a substrate (**Figure 14F**). The IC_{50} in a cell-based assay measuring reduction of NE and CatG activities in U937 cells constitutively expressing NE and CatG was found to be ~7 nM, which demonstrated the cell-permeable nature of

IcatC (Korkmaz et al., unpublished). The compound was found to be selective over cathepsins H, K, L, S, NSPs and DPP4 (Guarino, et al., 2017).

An almost complete inactivation of PR3 and CatG was observed in PLB-985 and HL60 cells, which mimic neutrophilic precursors at different stages of neutrophil maturation (Hamon, Legowska, et al., 2016b). However, the amounts of PR3 were not lowered by pharmacological CatC inhibition using IcatC, in contrast to what was observed in neutrophils from PLS patients (Hamon, Legowska, et al., 2016a; Methot, et al., 2007). This observation could be due to the use of immortalized cell lines, the protease content of which largely differs from that of bone marrow precursor cells. To test this hypothesis, Guarino et al., investigated the stability of NE zymogens in neutrophil progenitors isolated from human bone marrow, which were pulse-chased up to 5 days in the presence of IcatC (Guarino, et al., 2017). The cells incubated with or without IcatC were clearly differentiated by 24 h and by 96 h, identified by more than 70% band cells and segmented cells. Pulse-chase experiments in the presence of IcatC showed that the NE zymogen was cleaved into low molecular weight fragment before disappearing completely during differentiation. However, the constitutive secretion of the NE zymogen by bone marrow precursor cells was not altered by IcatC treatment. Since NE zymogen is secreted into the medium to the same extent in the presence and absence of the CatC inhibitor and in PLS patients (Sorensen, et al., 2014), degradation of the NE zymogen most likely occurs in nascent azurophilic granules and not in the endoplasmic reticulum/Golgi apparatus. Altogether, these data support the proposal that zymogens are degraded during the time of granule formation and utilization before the neutrophils are released into the circulation.

In recent work, a special focus was given to PR3 that is exposed at the neutrophil surface, and functions as a main antigen for anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune vasculitis, such as granulomatosis with polyangiitis (GPA, formerly Wegener's disease) (Jenne, et al., 1990). Thus, pharmacological CatC activity blockade in patients with GPA could be of major relevance for future clinical studies. Seren et al., studied PR3 in neutrophils from 21 PLS patients and showed a largely reduced, but still detectable (0.5-4%) PR3 activity when compared to healthy control cells. The PR3 membrane expression on activated PLS neutrophils was diminished by approximately 80% as shown by flow cytometry. The effect of IcatC on PR3 expression in normal neutrophils using a

$CD34^+$ hematopoietic stem cell model was evaluated. Human $CD34^+$ hematopoietic stem cells from umbilical cord blood treated with IcatC during neutrophil differentiation over 10 days revealed almost complete abrogation of membrane PR3 and cellular PR3 as well as of the PR3 proteolytic activity. Neutrophil differentiation was not compromised in the presence of IcatC (Seren et al., unpublished).

Strongly reduced NSP protein abundance and activity after pharmacological CatC inhibition using IcatC in both neutrophil cell models was observed. These results are the first ever showing that pharmacological inhibition of CatC can result in the elimination of the zymogens of elastase-like proteases as observed in PLS patients.

Pharmacokinetic analysis in mice showed that IcatC was retained in the bone marrow and reached sufficient tissue levels for CatC inhibition. Subcutaneous administration of IcatC in two doses in a mouse model of rheumatoid arthritis induced by anti-collagen antibodies exhibited statistically significant anti-arthritic activity, with sustained reductions in mean total arthritis scores, mean rear paw arthritis scores and mean rear paw thickness (Korkmaz & Pedersen, unpublished). The study clearly demonstrated that an effective but not complete inhibition of CatC resulting in a 60-80% reduction of NSP activities was sufficient to counteract the induced arthritis. This example indicates that a therapeutic effect can be obtained without complete elimination of the NSPs, i.e. without coming close to a CatC knock-out situation.

The inhibition of NE and PR3 by long-term subcutaneous administration of IcatC (4.5 mg/kg; twice daily; 12 days) was confirmed in a non-human primate. Inhibition of CatC resulted in almost total elimination of NE and PR3 in white blood cells. Blood neutrophils lacking NSP activities were still recruited to the lung following a lipopolysachharride-induced airway inflammation (Guarino, et al., 2017). These preclinical results demonstrate that the disappearance of NSPs as observed in PLS patients can be achieved by pharmacological inhibition of bone marrow CatC. Such a transitory inhibition of CatC might thus help to rebalance the protease load during chronic inflammatory diseases, which opens new perspectives for therapeutic applications in humans.

Cyanamide based inhibitor: Cyanamides constitute another interesting group of nitrile-based inhibitors of CatC. In these compounds, the nitrile group is bound to the nitrogen of a saturated

heterocycle and their peptidic character is significantly reduced. Firstly, a series of N-cyclopyrazolidine-based CatC inhibitors, consisting mostly of aryl, benzyl and biaryl carbamates of N-cyclopyrazolidine was developed (D. I. Laine & Busch-Petersen, 2010). Compound **11** is an example from this series (**Figure 15**). Next, patent applications covered the compounds based on the 1-cyano-3aminopyrrolidine scaffold. The first one described 138 compounds in which the 3-amino group was functionalized to form phenyl sulfonamide. 2,5-substituted phenyl rings were preferred with Cl, Br or OMe in position 2 and substituted amines used in position 5. Compound **12** is a representative example. Another patent explored 1-cyano-3-aminopyrrolidine sulfonamides with the alkylated sulfonamide nitrogen (compound **13**). A different application reported 1-cyano-3aminopyrrolidine sulfonamides, in which the phenyl ring was replaced by other heteroaromatic groups such as thiophene, benzothophene or quinolone (compound **14**). The last application covered the examples of compounds in which the 5-position of the pyrrolidine ring was substituted with a functionalized methyl group, such as compound **15**.

In 2010, GlaxoSmithKline pursued the work on cyanamide-based CatC inhibitors with a study of 3-substituted pyrrolidine nitriles (D. Laine, et al., 2011). The starting point of the study was a 3-phenethyl derivative (compound **16**), which displayed strong inhibitory potency against CatC, but no selectivity over other cathepsins. In order to examine different linkers between the 5-membered ring and the phenyl group, a variety of 3-substituted pyrrolidine nitriles were prepared and studied. As a result, compound **17** with a sulfonamine moiety was identified to be a strong inhibitor, slightly superior to the analogues containing amide bonds or urea as a linker. Compound **17** was selective over other cysteine cathepsins, thus it was further optimized. Different substituents were tested in position 2 and 5 of the phenyl ring and a dibromo derivative displayed the highest inhibitory activity and good selectivity. Despite the fact that the compound was stable in human plasma, it exhibited high plasma turnover in rats. Hence, another modification was introduced for the use in *in vivo* models in rodents. The addition of a (5S)-methyl group to the dibromo derivative yielded compound **18 (Figure 13D** and **Figure 15)**, which displayed high potency against CatC (pIC_{50} of 8.5), first-rate selectivity over other cathepsins, as well as good stability in rat plasma (D. Laine, et al., 2011). Compounds **11-18** are shown in **Figure 15**.

Laine et al., evaluated compound **18** in a cigarette smoke mouse model following intranasal administration (30 mg/kg) (D. Laine, et al., 2011). The increased proteolytic activity of CatC was observed in lung homogenates. The level of CatC was significantly reduced by intranasal administration of compound **18**.

Nitrile derivatives seem to be the most promising class of CatC inhibitors as cyano groups were successfully used as an electrophilic probe in the Novartis antidiabetic drug vildaglipin (Galvus®), which is a DPP4 inhibitor. Among the inhibitors of cysteine cathepsins, only aminoacetonitrile-based CatK inhibitors reached advanced phases of clinical trials as drug candidates for the treatment of postmenopausal osteoporosis (Duong, 2012). Nevertheless, as of 2010, over 30 nitrile-containing pharmaceuticals were on the market, with more than 20 additional nitrile-containing leads in clinical development (Fleming, Yao, Ravikumar, Funk, & Shook, 2010), which proves that it is possible to develop successful nitrile-based inhibitors.

6.2 Irreversible CatC inhibitor

GSK2793660 ((S,E)-4-amino-N-(1-(indolin-1-yl)-6-methyl-1-oxohept-2-en-4-yl)tetrahydro-2H-pyran-4-carboxamide hydrochloride), a 4-amino-tetrahydropyranyl-4-carboxylic acid derived dipeptide, is an irreversible covalent α,β-unsaturated amide based CatC inhibitor. GSK2793660 is a potent and selective CatC inhibitor with an IC_{50} value of ~1 nM and a k_{inact}/K_i value of 9.0 x 10^4 $M^{-1}s^{-1}$ (Miller, et al., 2017).

Miller et al., evaluated the safety, tolerability, pharmacokinetics and pharmacodynamics of single oral ascending doses of GSK2793660 (from 0.5 to 20 mg), and of repeat oral doses of GSK2793660 (12 mg) in healthy male subjects for 21 days (Miller, et al., 2017). GSK2793660 is the second CatC inhibitor evaluated in humans. Due to the irreversible nature of GSK2793660 it was possible to evaluate the CatC activity in whole blood in the randomized and placebo-controlled phase 1 study. It was shown that single oral doses of GSK2793660 were able to inhibit ≥90% of whole blood CatC activity within 1-3 h of dosing on day 1. The impact of GSK2793660 on whole blood NSP activity was evaluated following 21 days repeat dosing with placebo or GSK2793660 12 mg once daily. A fair inhibition of blood NSP activity of approximately 20% was observed: NE activity was

reduced by 7-47%. CatG and PR3 activity were reduced by up to 47% and 37%, respectively. However, for all of the measurements, there was fluctuation across the time points such that no consistent relationship between time and enzyme activity levels could be determined (Miller, et al., 2017).

The development of epidermal desquamation on palmar and plantar surfaces after 7-10 days dosing with GSK2793660 was observed in 7 of 10 subjects receiving repeated doses of GSK2793660. This desquamation was intense but reportedly caused no symptoms and thus cannot be considered severe. No other clinically important safety findings were reported. However, preclinical toxicology studies conducted both on rats and dogs for up 3 months with high doses of GSK2793660 did not reveal skin manifestations. The epithelial desquamation observed in this clinical trial has some similarities to the phenotype of CatC-deficient patients and it was hypothesized that CatC or one of its target proteins has a previously unidentified role in the maintenance and integrity of the epidermis. The chemical structure of GSK2793660 contains an alpha-, beta- unsaturated carboxamide. This is a well-known structure for conjugation with cysteinyl groups of proteins and peptides, and therefore this unsaturated group is considered a structural alert in drug metabolism-safety terms (Sardi, et al., 2013; Winther & Thorpe, 2014). However, this property of GSK2793660 has not been investigated in relation to the reported drug-related adverse event.

7. CONCLUSIONS

To date, the most precisely described function of CatC is to activate a variety of immune cell-derived serine proteases. However, the list of CatC targets has not yet been completed and a number of questions remains to be investigated in order to fully understand the biological significance of this protease. For example, its functions in the peripheral tissues has not been explained until now. Little is known about the physiological regulation of CatC: apparently different cysteine proteases can activate its proform, depending on the cell and tissue. The fate of CatC and its putative control by natural inhibitors also remains enigmatic. Targeting CatC by exogenous inhibitors will help to delineate its function in all physiological mechanisms that involve the participation of its substrates and will

provide therapeutic means to fight inflammatory diseases, where it's direct or indirect implication has been clearly established. The non-serious skin manifestations observed in clinical trials related to the administration of a CatC inhibitor demands further clinical developments. Curiously enough however, genetic deficiency in CatC activity, as observed in PLS, does not compromise the survival of patients although it has clearly visible pathophysiological consequences. Prolonged pharmacological inhibition of bone marrow CatC in preclinical trials results in inhibition and elimination of elastase-like proteases in blood neutrophils as observed in PLS patients. Mimicking the genetic deficiency in PLS neutrophils by pharmacological inhibition of CatC in bone marrow provides an attractive strategy for researching neutrophil-driven chronic inflammatory or autoimmune diseases.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1: Regulation of the expression and activity of cysteine cathepsins. A) Diverse checkpoints (transcription, translation, and maturation) govern the expression, maturation and activity of cysteine cathepsins. Regulatory sites are found in the promoter region of some cathepsin genes, while epigenetic regulation has been very poorly investigated. Cysteine cathepsins are synthesized as (pre) proforms (zymogens) that could be glycosylated and phosphorylated. Pro-cathepsins are further converted to mature active forms by autocatalytic and/or heterocatalytic mechanisms leading to the release of the proregion (propeptide). B) Post-translational regulation. The activity of mature cathepsin is modulated by endogenous inhibitors (cystatins, propeptides) or by various proteolytic machineries. Supplementary molecular modifications and posttranslational regulatory systems participate in the control the proteolytic activity of cysteine cathepsins (e.g. metal/ion binding, negatively charged glycosaminoglycans, redox potential, oxidative stress, pH and compartmentalization).

Figure 2: 3D Structure of processed CatC monomer. A) Structures of pro-CatC and mature CatC. **B)** Ribbon representation of 3D structure of processed CatC monomer (PDB 2DJF, (Molgaard, et al., 2007)). Exclusion domain, propeptide, heavy chain and light chain are colored in red, black, green and blue, respectively. The image was created with Yasara (http://www.yasara.org).

Figure 3: 3D structure of functional tetrameric CatC. A) Ribbon representation of 3D structure of hometetrameric CatC (PDB 2DJF, (Molgaard, et al., 2007)). The active sites are located at the four corners of the tetramer. **B)** Solvent accessible surfaces of CatC with positive or negative electrostatic potential are colored dark blue and red, respectively. The images were created with Yasara.

Figure 4: Schematic image of the CatC substrate binding site in complex with a peptide substrate. The Asp1 of the exclusion domain and the catalytic Cys234 are shown in red and green, respectively.

Figure 5: Dermatological and dental features of PLS patients. A) Top, palmoplantar keratoderma in PLS showing the typical diffuse, red, scaling in a child (*left*) and the stippled appearance on the sole of an adult (*right*). Bottom, unusually severe transgrediens involvement, with psoriasiform appearance, in a 6-year old child with PLS. **B)** Top, patient with PLS in the primary dentition demonstrating gingival inflammation, evidence of infection, migration of teeth, and plaque

accumulation in most severely affected teeth. Bottom, patient aged 13 years with PLS demonstrating extensive bone loss around first permanent molars and incisors.

Figure 6: Schematic to show genomic location and organization of CatC gene, transcript and translated polypeptide. A) Genomic location of *CTSC* in chromosome band 11q14.2. **B)** Genomic organization of the *CTSC* with exons indicated by numbering. **C)** *CTSC* transcript with exon derivation indicated in the boxes and amino acids encoded corresponding to the key elements of the polypeptide chain indicated below. **D)** *CTSC* translated polypeptide. SP, signal peptide. The amino acid numbering for these regions is indicated below.

Figure 7: Immune reactive levels of NE, PR3 and CatG in PLS blood samples. Western-blotting of white blood cell lysates from PLS and healthy controls using antiNE, antiPR3 and antiCatG antibodies.

Figure 8: Representative model of proposed events, secondary to CatC deficiency underlying pathogenesis of aggressive periodontitis in PLS. Bacteria and their products penetrate tissues during infection (a). Stimulation of circulating host immune cells e.g. macrophages, which release chemokines/cytokines e.g. CXCL8, MIP1α (β). Neutrophils recruited from circulation (c). Neutrophils kill bacteria at infection site (d). NSP action contributes to a coordinated immune response; regulation of neutrophil recruitment and inflammatory response (e). In PLS neutrophils, a lack of CatC results in a lack of NSP activity resulting in absence of NET release increased ROS and pro-inflammatory mediators (f). NSP deficiency results in failure to deactivate CXCL8 and MIP1α and thus in the relentless neutrophil recruitment and ineffective killing, which underlies the host tissue destruction and eventual tooth loss (g).

Figure 9: Activation of pro-chymase by CatC. The left and right panels show structures prepared with the iCN3D structure viewer from crystal-based structures of human pro-chymase (PDB 1NN6 (Reiling, et al., 2003)) and mature chymase (PDB 1PJP (Pereira, et al., 1999)) inactivated by a peptidic inhibitor (shown in cyan) in the active site. CatC removes surface-exposed pro-dipeptide Gly-Glu, thereby generating neo-N-terminus Ile-Ile-Gly-Gly-Glu-Cys (blue chain), which dives into the interior

of the protease, resulting in activation-associated structural changes, including major movement of a loop (shown in red) near the active site.

Figure 10: NSPs, ANCA-induced inflammation, and autoimmunity in AAV. (1) PR3 is presented on the cell surface of viable neutrophils, either by direct membrane insertion or by CD177. PR3-ANCA, by binding to its target, initiates neutrophil signaling and activation. (2) ANCA-stimulated neutrophils release NETs that provide a scaffold for alternative complement pathway activation. The resulting C5a interacts with the activating C5a receptor on several cell types, thereby accelerating inflammation. (3) ANCA stimulate IL-1β generation in monocytes and neutrophils that promotes NCGN. NSPs, most likely PR3 or a PR3/NE combination, contribute to the processing of pro-IL-1β into mature IL-1β. (4) NSPs are released from ANCA-activated neutrophils and acquired by neighboring cells (e.g. endothelial cells) where they cleave intracellular substrates leading to endothelial damage. (5) Enzymatically active PR3 on apoptotic neutrophils inhibits phagocytosis by macrophages and increases the production of several pro-inflammatory cytokines and chemokines. This milieu, in the presence of PR3-ANCA, causes pDCs to polarize $CD4^+$ T cells towards Th17 cells. (6) Reactivated transcription of NSPs occurs in neutrophils obtained from patients with active AAV.

Figure 11: Tracking NSPs as biomarkers for pharmacological CatC inhibition in laboratory, in preclinical and clinical studies.

Figure 12: Chemical structures of nitrile-based inhibitors of CatC derived from Abu-Bip-CN.

Figure 13: X-ray structures of nitrile-based inhibitors (Abu-Bip-CN, compound 2 and compound 18) in complexes with CatC. Reaction of the nitrile function with the active site cysteine 234 resulting in the formation of a reversible thioimidate complex is shown in A. Inhibitor and CatC are colored cyan and ochre, respectively in B-D. Oxygen and nitrogen atoms are shown respectively in red and in blue. Sulfur and brome atoms are colored green. PDB codes: 4cdc (Abu-Bip-CN), 4cdf (compound **2**) (Furber, et al., 2014), 3PDF (compound **18**) (D. Laine, et al., 2011). The images were created with Yasara. The structures of compounds are shown in Figures 12 and 18.

Figure 14: Chemical structures of nitrile-based inhibitors of CatC with β-(2-thienyl)-L-alanine at P2 position (A-D) and chemical structure of IcatC (XPZ-01) (E).

Figure 15: Chemical structures of cyanamides exhibiting inhibitory activity against CatC.

Table 1. Main characteristics of CatC

EC Number	EC 3.4.14.1
Classification of the MEROPS database	Family C1A of clan CA
Gene name and gene locus	*CTSC*,11q14.1-q14.3
loss of function mutations	Papillon-Lefèvre syndrome (OMIM: 245000) and the related Haim-Munk syndrome (OMIM: 245010)
Tissue specificity	Ubiquitous Highly expressed in lung, kidney, placenta and immune cells. Detected at intermediate levels in colon, small intestine, spleen and pancreas
Subcellular location	Granules
Endogenous activator	Cathepsin L, S, K, V, F
Molecule processing	Signal peptide Exclusion domain Propeptide Heavy chain Light chain
Amino acid modification	Glycosylation and disulfides
number of glycosylation site	4 (3 on exclusion domain, 1 on heavy chain)
number of disulfide bridge	5 (2 on exclusion domain, 3 on heavy chain)
Molecular mass	Mature CatC (homotetramer) 200 kDa
Available crystallographic structures	Free humCatC (PDB code: 1K3B) Free ratCatC (PDB code: 1jqp) humCatC in complex with an inhibitor (PDB codes: 2DJF, 2DJG, 4cdc, 4cdd, 4cdf, 4cde, 3PDF)
Optimal pH activity	6 (active in the pH range between 3.5 and 8.0)
Substrate specificity	Activation by chloride ion Stop sequence: R/K in P2; P in P1 or P1'; I in P1
Inhibitors chemical	Chemical and protein Diazomethyl ketone O-acyl hydroxamate Vinyl sulfone E64c-based inhibitors Semicarbazide Nitrile Organophosphonate
natural	Stefin A and B, cystatin C and F, Olfactomedin
Biological functions	Activator of granule serine proteinases: NE, PR3, CatG, NSP4, chymases, tryptases, granzyme A, granzyme B Lysosomal degradation of peptides

Table 2. Relevant benchmarks in research on CatC using knock-out ($CatC^{-/-}$) mice

Year	Benchmark	References
2002	*EXPERIMENTAL ACUTE ARTHRITIS* · CatC identified as NSP activating protease · Normal granulocyte development showed in $CatC^{-/-}$ mice · Resistance of $CatC^{-/-}$ mice to induction of experimental acute arthritis observed · Role of CatC in regulation of the development of acute arthritis	(Adkison, et al., 2002)
2004	*SEPSIS* · Protection of $CatC^{-/-}$ mice from death due to sepsis observed · Mast cell CatC identified as a key regulator of survival from septic peritonitis	(Mallen-St Clair, et al., 2004)
2005	*COLLAGEN-INDUCED ARTHRITIS (CIA)* · Protection of $CatC^{-/-}$ mice against the development of CIA observed · Role of CatC in a critical step in development of CIA attributed	(Hu & Pham, 2005)
2007	*ELASTASE INDUCED ABDOMINAL AORTIC ANEURYSM (AAA)* · Resistance of $CatC^{-/-}$ mice to elastase induced AAA observed · Role of CatC in neutrophil recruitment into diseased aorta	(Pagano, et al., 2007)
2008	*INFLAMMATORY RESPONSE TO SENDAI INFECTION* · Role of CatC in the recruitment of neutrophil following Sendai virus infection attributed · Chronic asthma phenotype diminished in $CatC^{-/-}$ mice	(Akk, et al., 2008)
2012	*ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY (ANCA)-ASSOCIATED NECROTIZING CRESCENTIC (NCGN)* · Protection of $CatC^{-/-}$ mice from ANCA-induced NCGN observed	(Schreiber, et al., 2012)
2014	*ATHEROSCLEROTIC LESION PROGRESSION* $CatC^{-/-}$ presented attenuated atherosclerotic lesion progression	(Herias, et al., 2015)

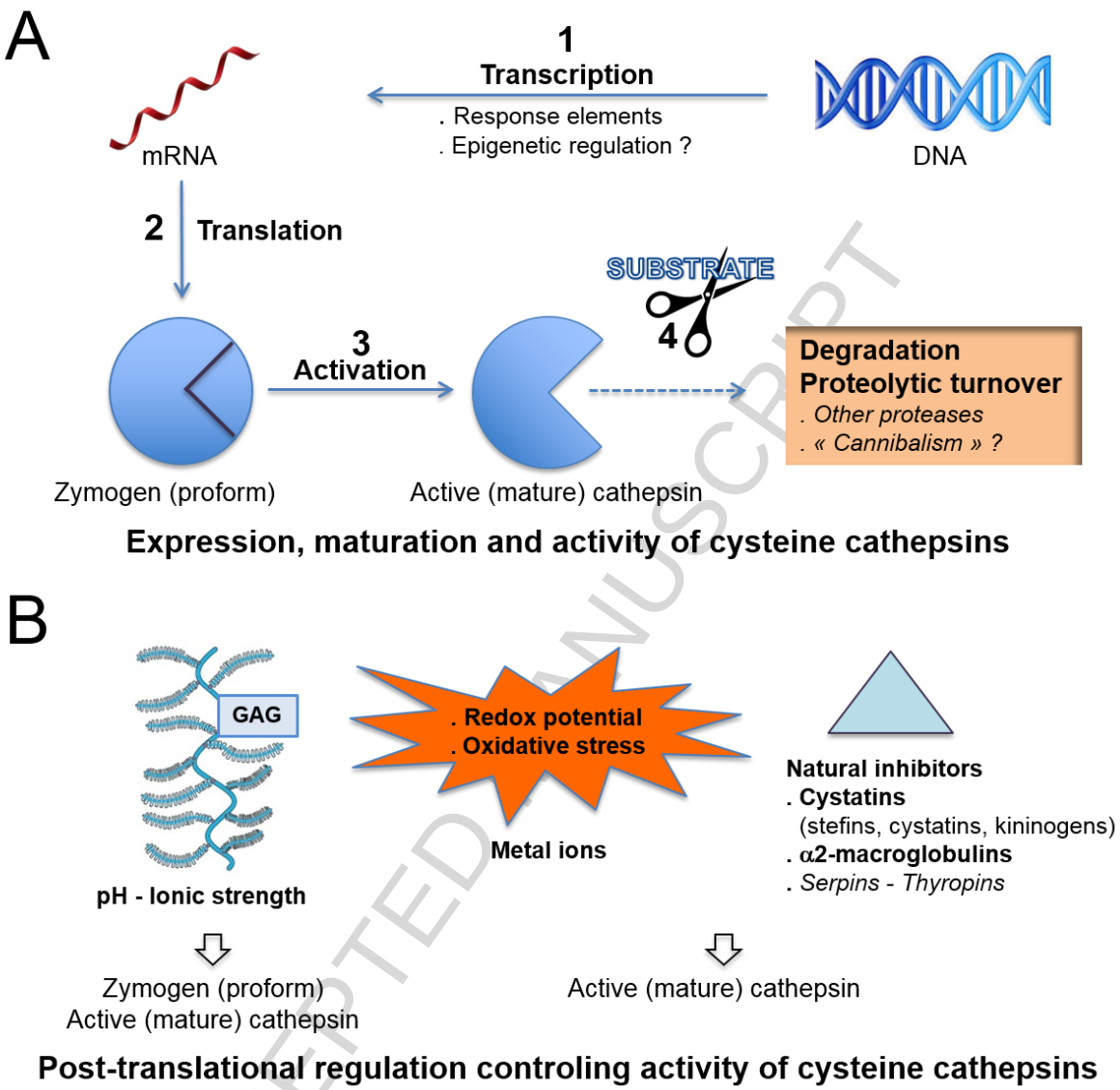
$CatC^{-/-}$ mice don't exhibit dental and skin manifestations observed in PLS patients.

Table 3. Propeptides and potential CatC-targeted activation sites of neutrophil and mast cell granule-associated serine proteases

Protease	Gene	Propeptide[a]
NE, human	*ELANE*	SE↓IVGGRR…
NE, mouse	*Elane*	SE↓IVGGRP…
Myeloblastin/PR3, human	*PRTN3*	AE↓IVGGHE…
Myeloblastin/PR3, mouse	*Prtn3*	SK↓IVGGHE…
NSP4, human	*PRSS57*	AQ↓IIGGHE…
NSP4, mouse	*Prss57*	SY↓IVGGHE…
CatG, human	*CTSG*	GE↓IIGGRE…
CatG, mouse	*Ctsg*	GK↓IIGGRE…
Mast cell chymase, human	*CMA1*	GE↓IIGGTE…
Mast cell chymase 5, mouse	*Cma1*	GE↓IIGGTE…
Mast cell chymase 1, mouse	*Mcpt1*	GE↓IIGGTE…
Mast cell chymase 4, mouse	*Mcpt4*	EE↓IIGGVE…
Tryptase αII, human	*TPSAB1*	APAPVQALQQAG↓IVGGQE…
Tryptase MCP-7, mouse	*Mcpt7/Tpsab1*	APGPAMTREG↓IVGGQE…
Tryptase βII, human	*TPSB2*	APAPGQALQRVG↓IVGGQE…
Tryptase MCP-6, mouse	*Mcpt6/Tpsb2*	APRPANQRVG↓IVGGHE…
Mastin MCP-11, mouse	*Prss34*	NTMPLTLDLGSGQGLVG↓IVGGCP…
Mastin, dog	*LOC448801*	NTMPLTLDLGSGQGLVG↓IVGGCP…
Tryptase γ, human	*TPSG1*	ISPDPGLRHEQVG↓IVGGCK…
Tryptase γ/TMT, mouse	*Tpsg1*	GCGHPQVSNSGSR↓IVGGHA…

[a]Proposed propeptide is to the left of arrow (↓), which indicates site of hydrolysis required to generate mature, active protease.

FIGURE 1



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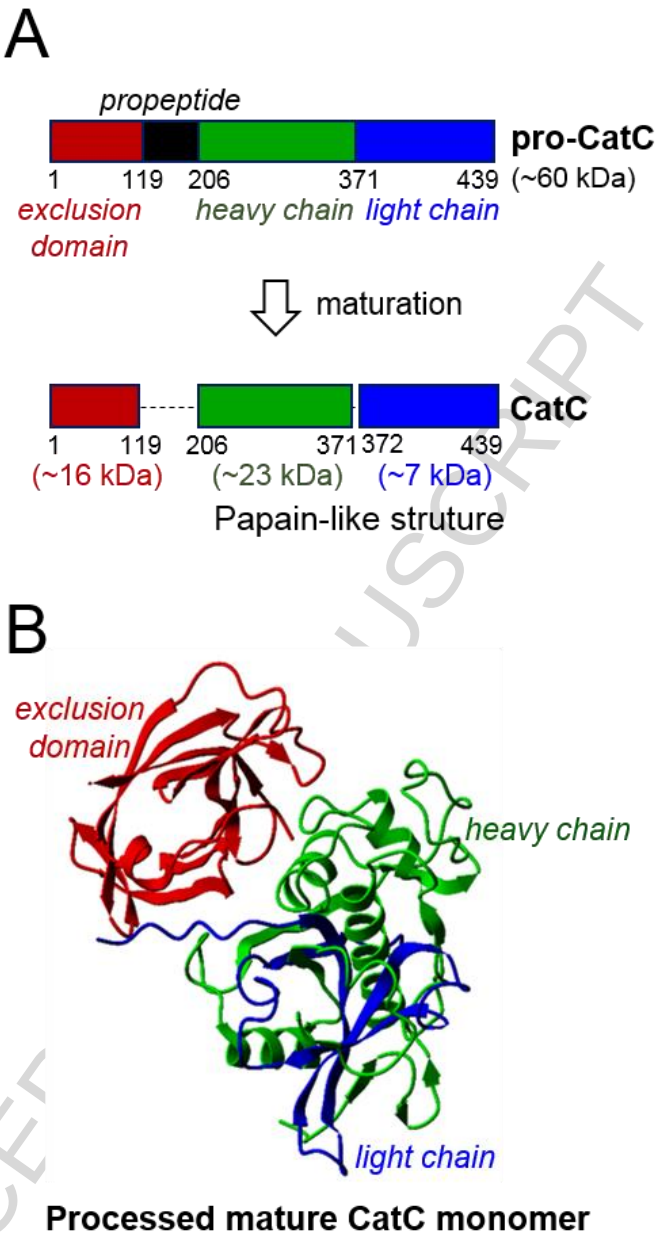

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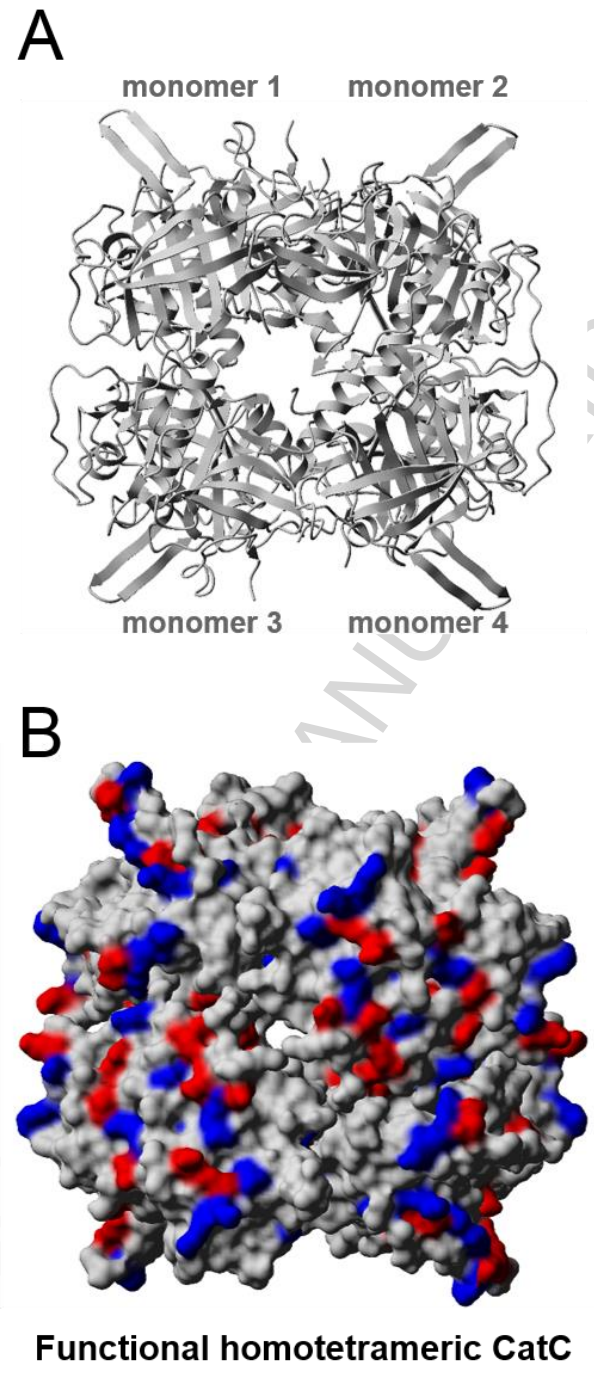
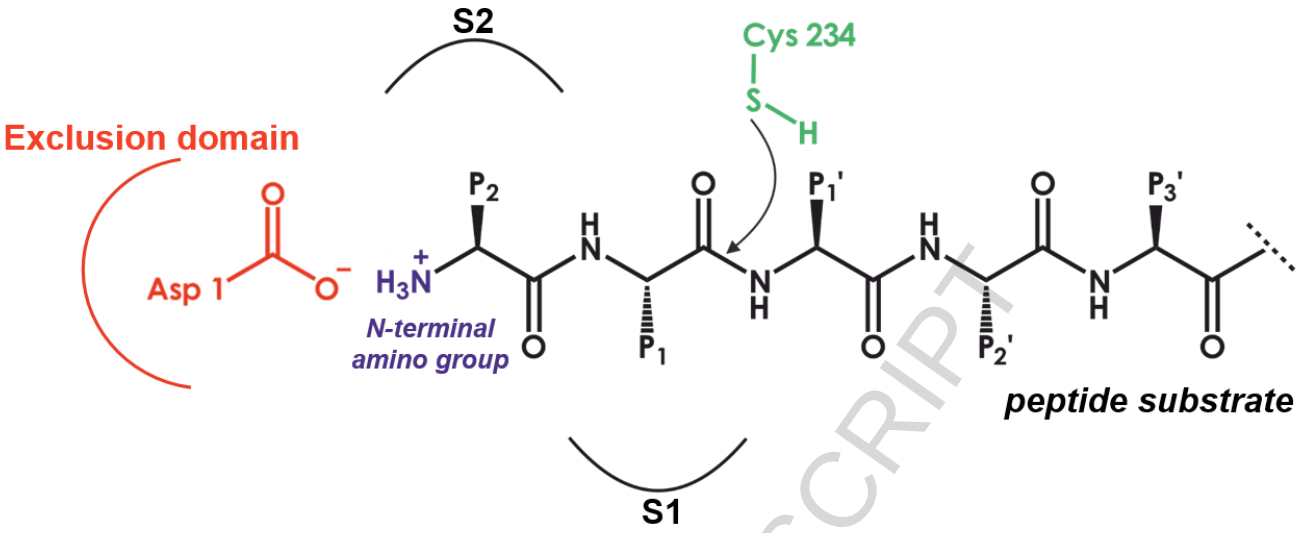


Functional homotetrameric CatC

FIGURE 4



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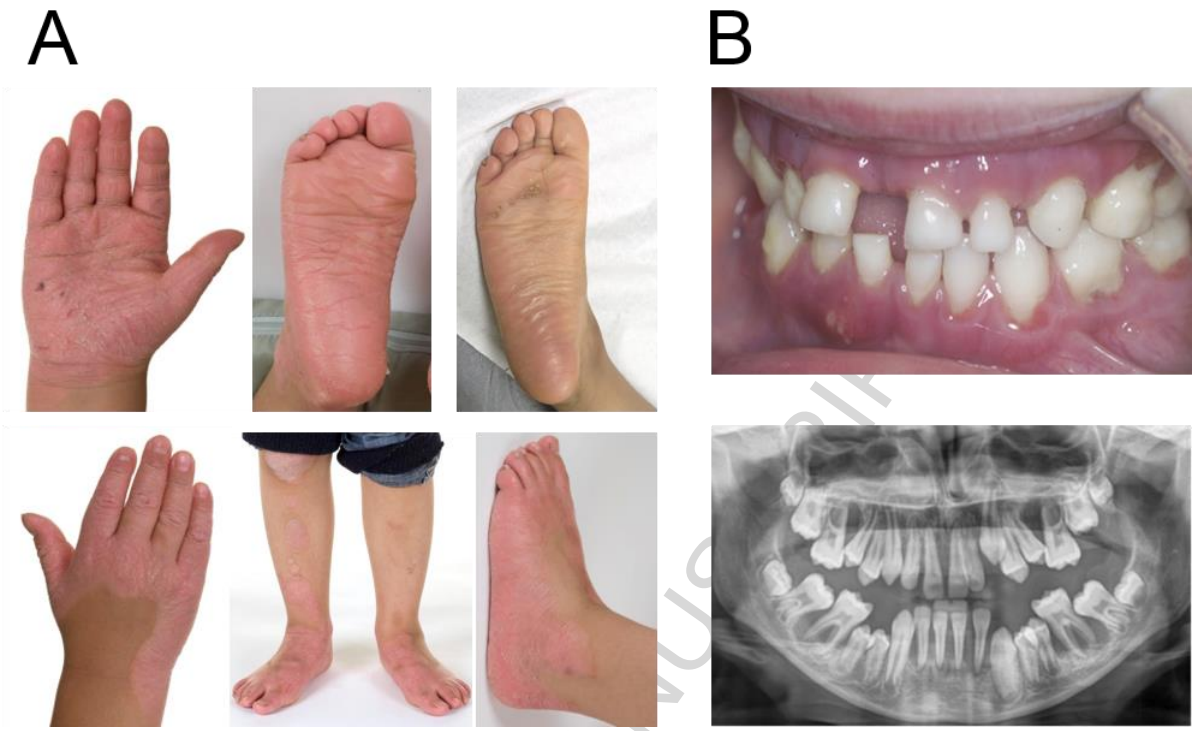

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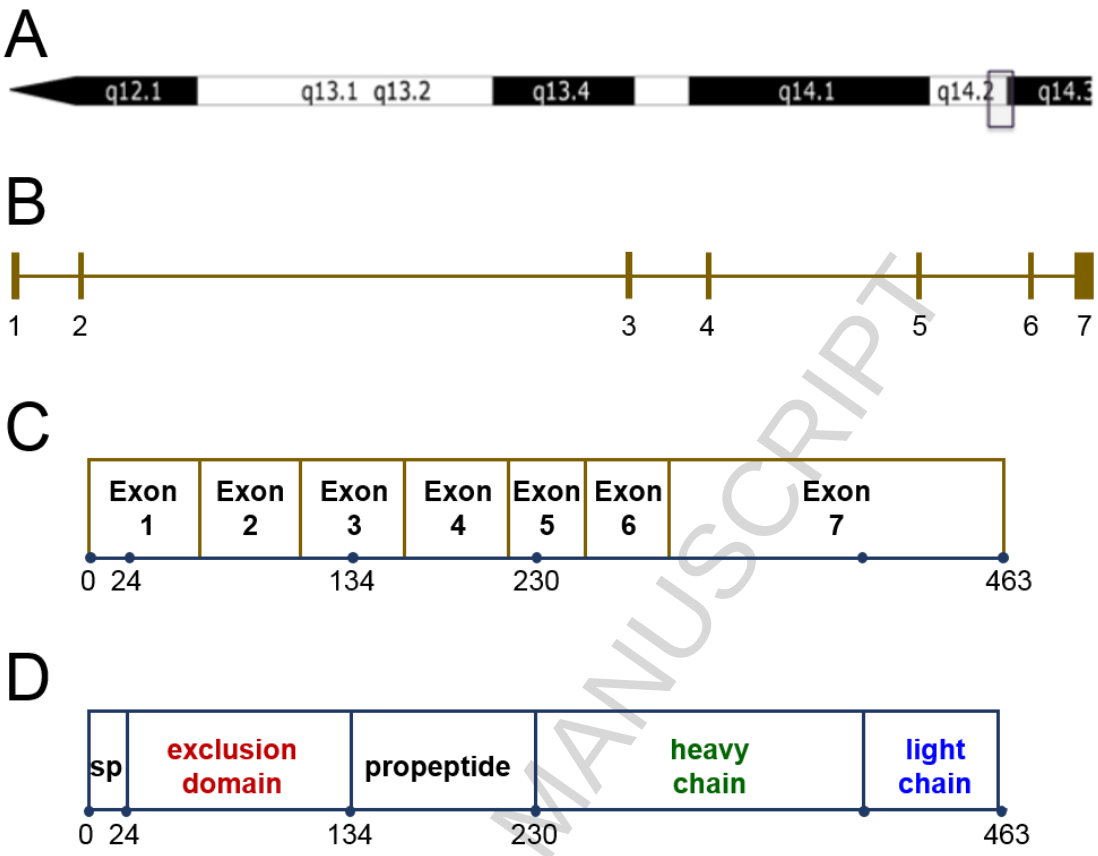

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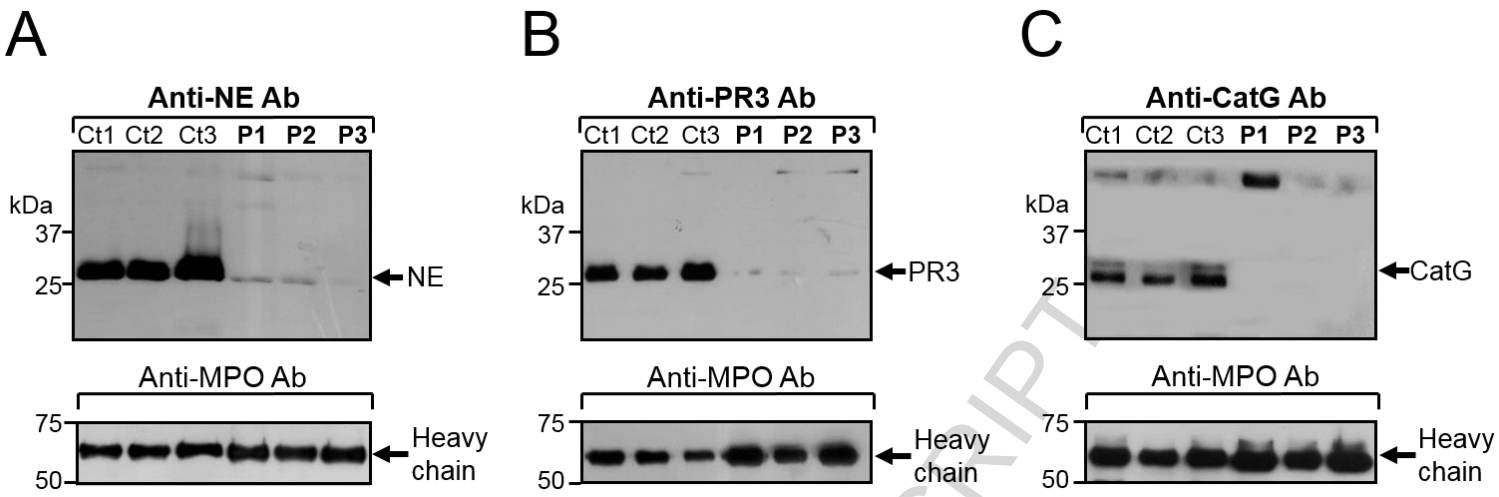

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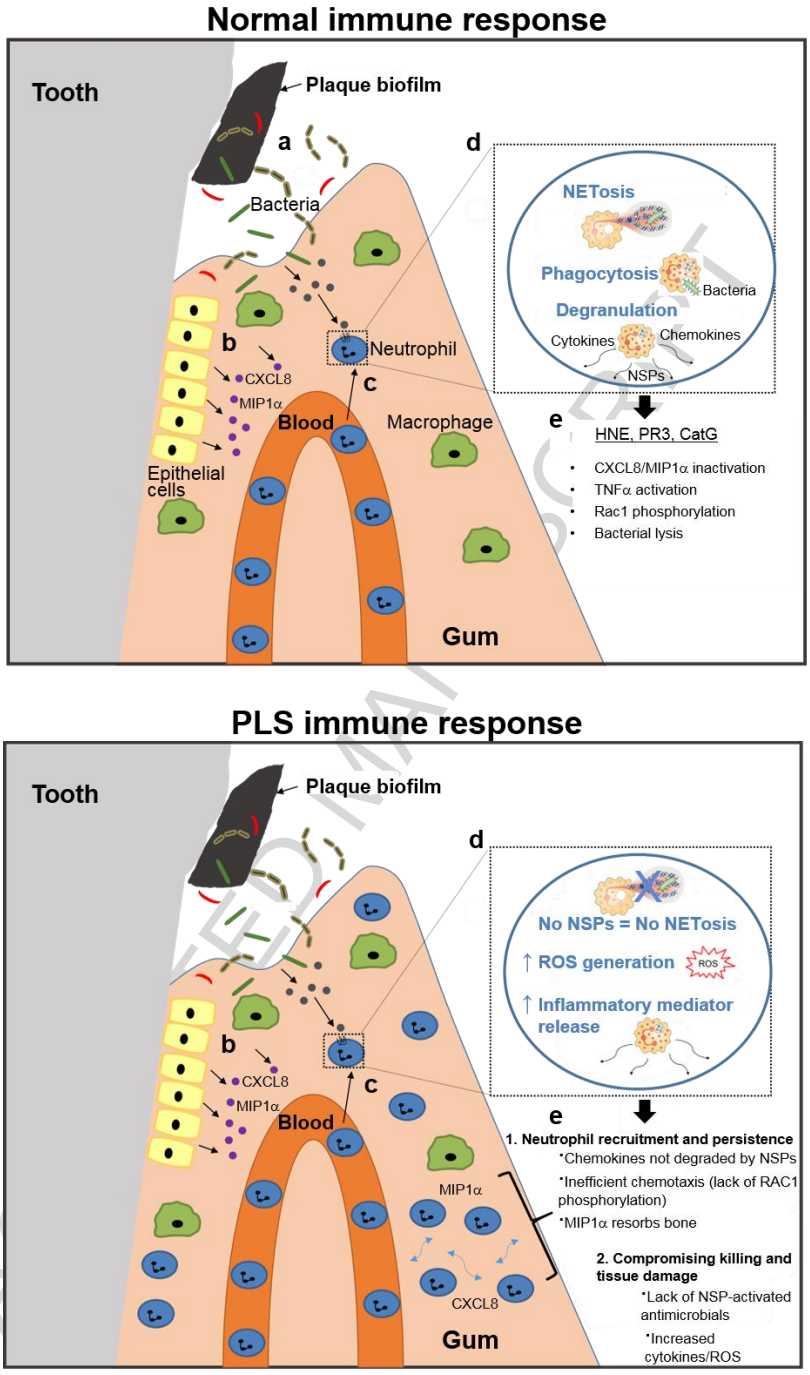

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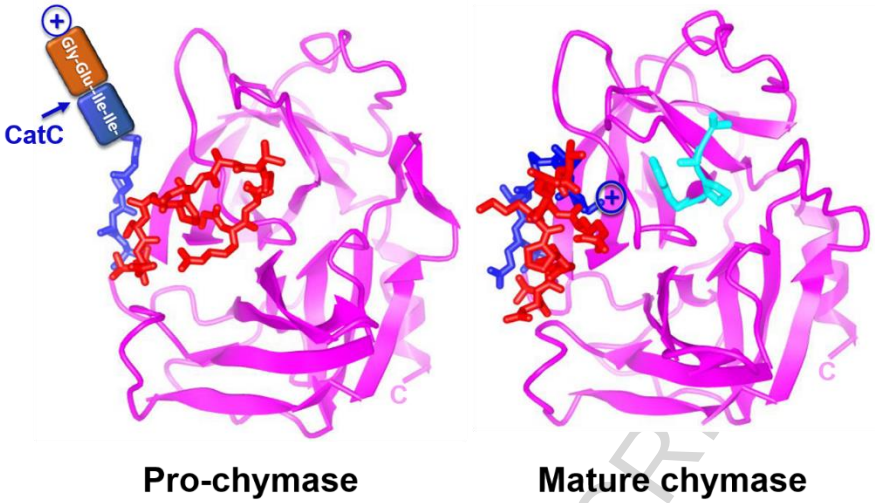

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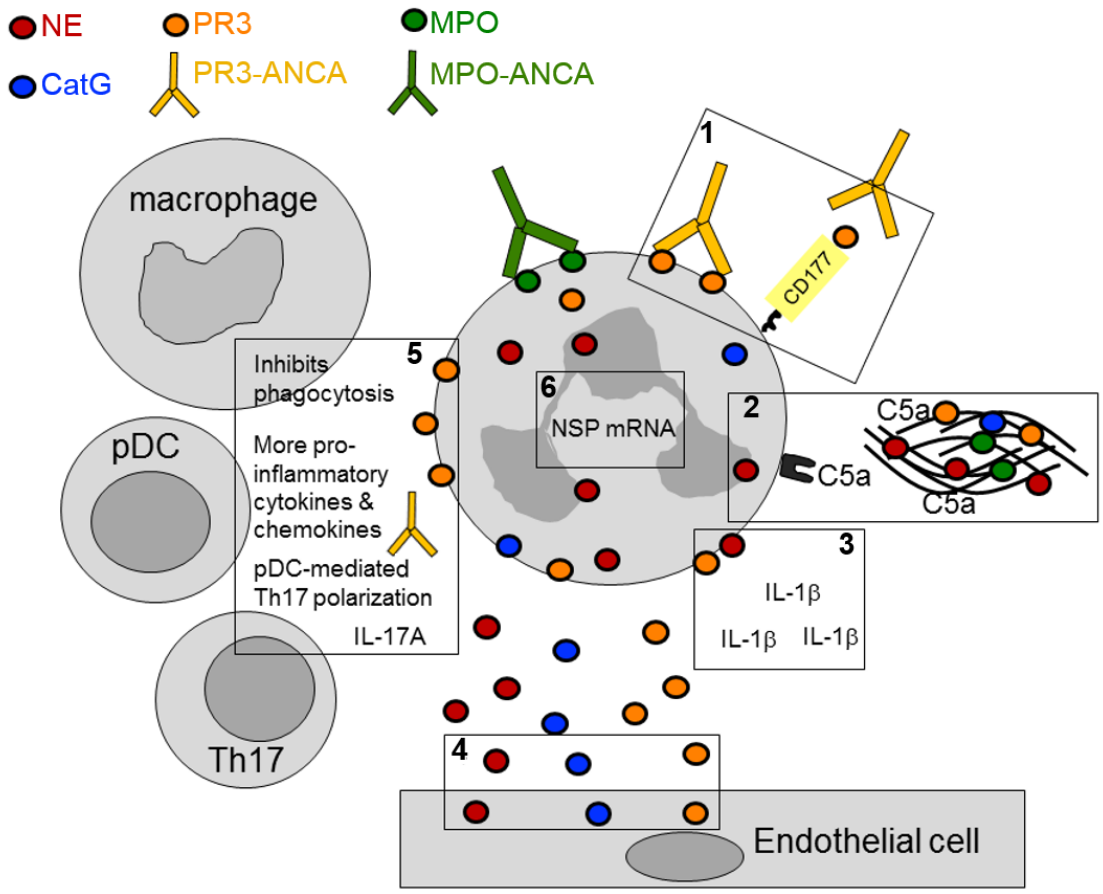

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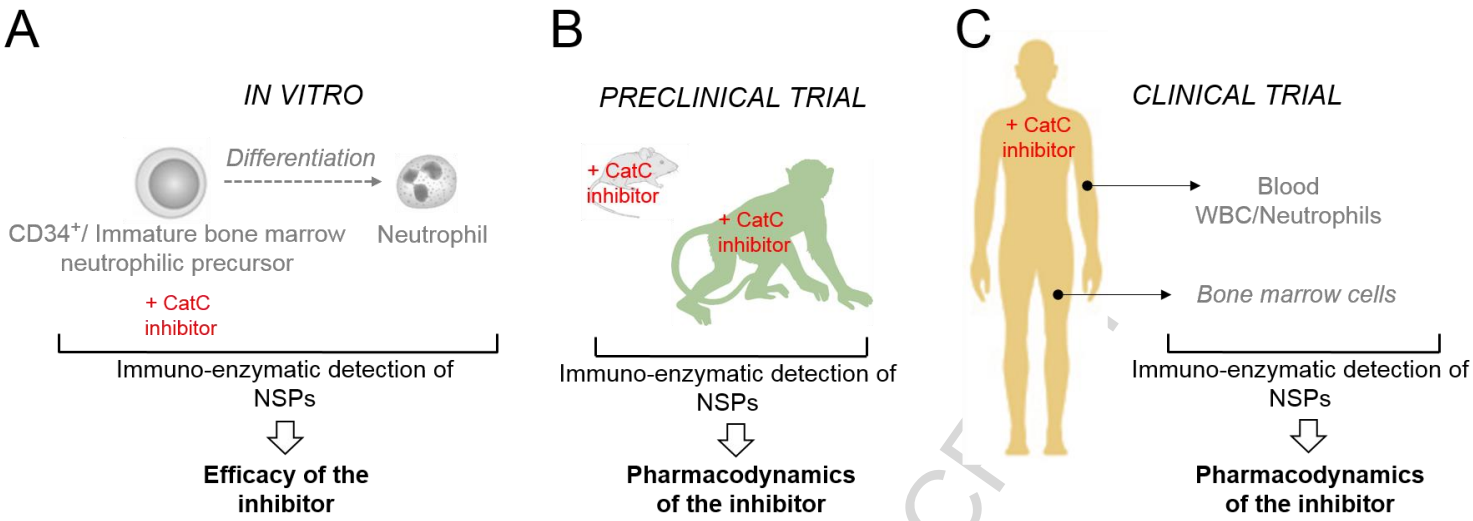

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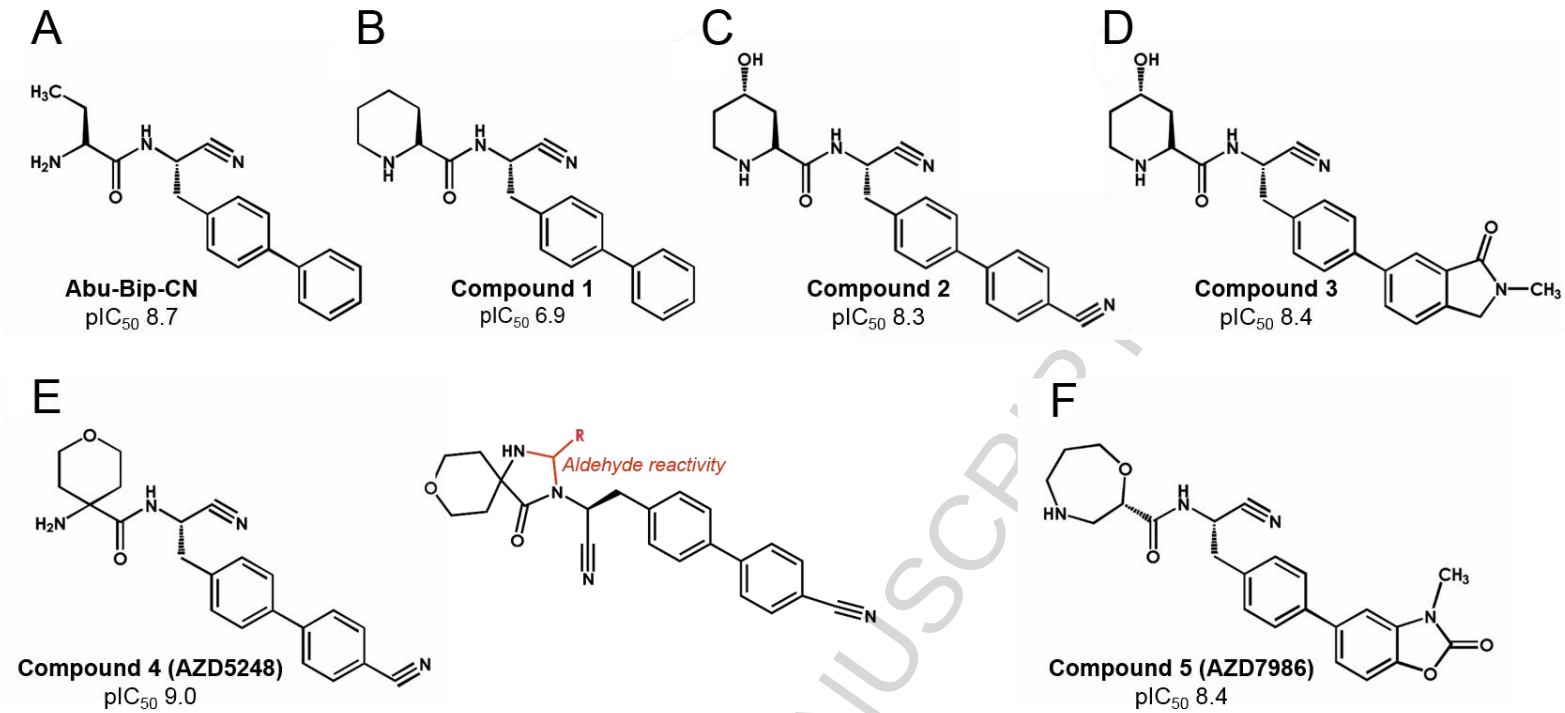

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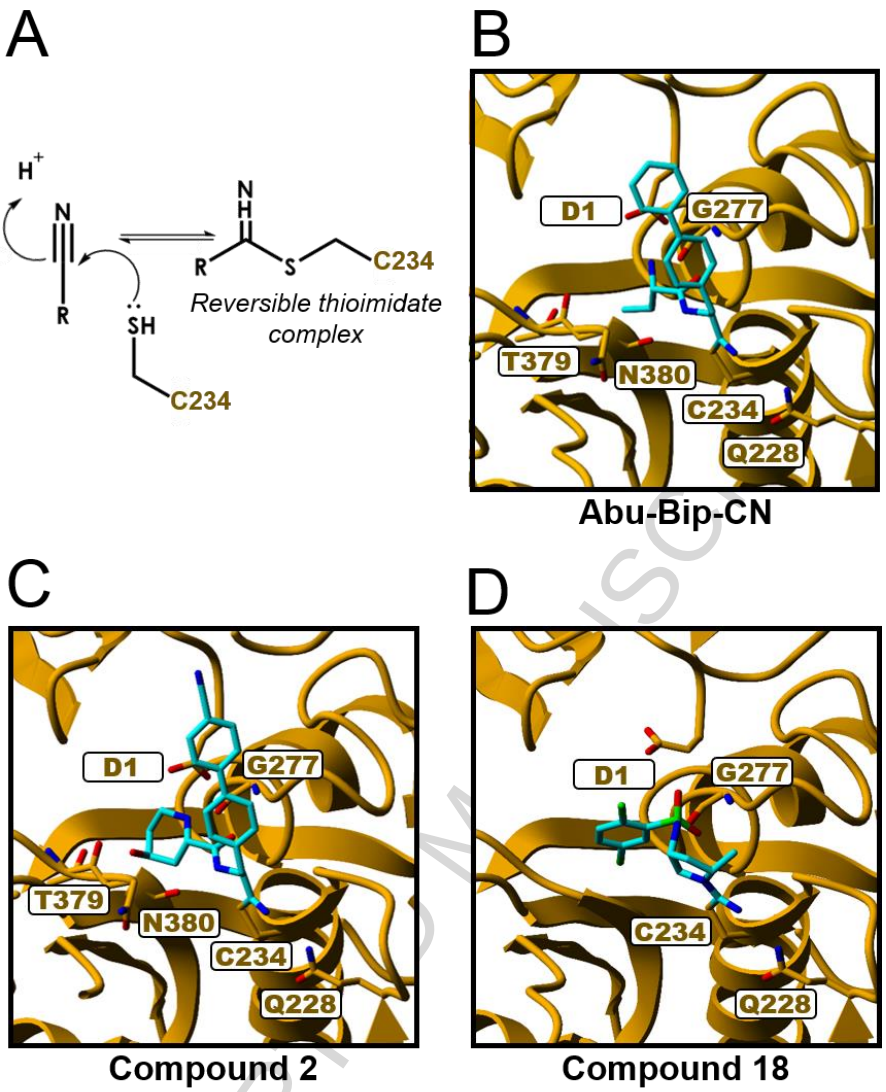

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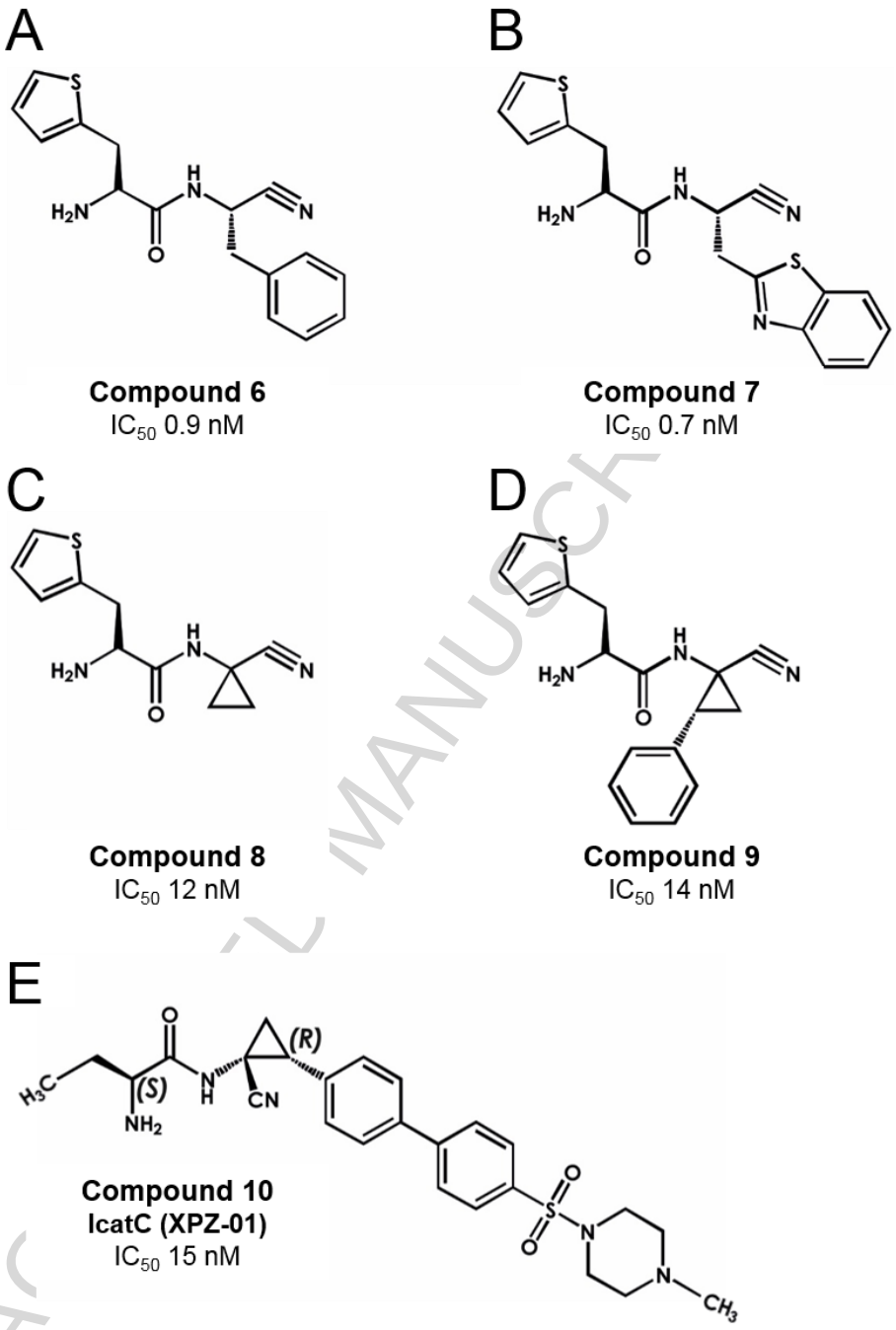

Figure 15